

Evidence of a Novel Event during Neuronal Death: Development of Competence-to-Die in Response to Cytoplasmic Cytochrome c

Mohanish Deshmukh and Eugene M. Johnson, Jr.*
Department of Neurology and
Department of Molecular Biology and Pharmacology
Washington University School of Medicine
St. Louis, Missouri 63110

Summary

Sympathetic neurons undergoing programmed cell death after nerve growth factor (NGF) deprivation are shown to exhibit a protein synthesis-dependent, BAX-dependent loss of cytochrome c from the mitochondria. However, cytoplasmic microinjection of cytochrome c was insufficient to induce cell death in NGF-maintained sympathetic neurons. In contrast, microinjection of cytochrome c rapidly induced a caspase-dependent death in NGF-deprived, *Bax*-deficient or NGF-deprived, cycloheximide-treated neurons. Cells needed to be deprived of NGF for 15–20 hr before they acquired competence to die with injection of cytochrome c. These data suggest that NGF deprivation induced the translocation of cytochrome c and another event, which we term as competence-to-die, that was independent of macromolecular synthesis and BAX function. Both these processes were required for neurons to undergo apoptosis.

Introduction

Programmed cell death (PCD) is an evolutionarily conserved and genetically regulated process by which cells commit suicide. Cells undergoing PCD exhibit characteristic morphological changes of apoptosis, including cytoplasmic shrinkage, plasma membrane blebbing, chromatin condensation, and DNA fragmentation (Kerr et al., 1972). PCD occurs extensively during development and may contribute to numerous pathological conditions, including stroke, spinal cord injury, and certain neurodegenerative diseases (Choi, 1996; Linnik, 1996; Stefanis et al., 1997). Deficiencies in PCD contribute to oncogenesis (Reed, 1997a).

Genetic and biochemical studies have implicated the caspases, Apaf-1, and the BCL-2 family of proteins as important regulators of PCD in mammalian cells. These are homologs of the *ced-3*, *ced-4*, and *ced-9* gene products, respectively, that are required for regulating PCD in *Caenorhabditis elegans* (Ellis et al., 1991). Caspases, which are cysteine proteases that cleave after aspartic acid residues, appear to be the main executors of apoptosis. In cells undergoing PCD, these proteases are activated by cleavage of their prodomain; activated caspases cleave specific cellular proteins and thereby cause cell death. Inhibition of caspases, either by cellular or virally encoded inhibitors, or by synthetic peptides,

prevents apoptosis in a variety of cell types (Salvesen and Dixit, 1997).

The BCL-2 family of proteins consists of both anti-apoptotic (e.g., BCL-2, BCL-X_L, and BCL-W) and pro-apoptotic (e.g., BAX, BAK, BAD, BIK, and BID) members. The ratio of antiapoptotic to proapoptotic BCL-2 family members within a cell may determine whether a cell will die after a death signal (Oltvai et al., 1993; Yang and Korsmeyer, 1996). Accumulating evidence suggests that the BCL-2 family proteins form or regulate the formation of channels on mitochondrial membranes (Schendel et al., 1998). The structure of BCL-X_L, having two central α helices surrounded by five amphipathic helices, is analogous to that of bacterial toxins such as diphtheria toxin and the colicins (Muchmore et al., 1996). Like the bacterial toxins, BCL-X_L (as well as BCL-2 and BAX) can insert into synthetic lipid vesicles or planar lipid bilayers and form ion-conducting channels (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). Whether these proteins form pores in mitochondrial membranes in intact cells and what signals regulate this activity are not known.

Recent reports have suggested a surprising function for cytochrome c in promoting apoptosis. First, cytochrome c is required for the formation of a complex that is sufficient to activate caspases in cell-free extracts (Liu et al., 1996; Kluck et al., 1997a). In vitro, binding of Apaf-1, procaspase-9, and cytochrome c, in the presence of dATP, results in the activation of caspase-9; activated caspase-9 presumably cleaves and activates other caspases and promotes subsequent apoptotic events (Li et al., 1997b). Second, cytochrome c translocates from the mitochondria to cytosol in cells in several models of apoptosis (Liu et al., 1996; Du et al., 1997; Kharbanda et al., 1997; Kluck et al., 1997a; Srinivasan et al., 1998). Third, microinjection of cytochrome c is sufficient to induce a caspase-dependent cell death in some cell lines (Li et al., 1997a).

Overexpression of the antiapoptotic BCL-2 or BCL-X_L protein prevents the translocation of cytochrome c in cells after a death-inducing stimulus (Kharbanda et al., 1997; Kluck et al., 1997a; Yang et al., 1997; Duckett et al., 1998; Srinivasan et al., 1998). In contrast, overexpression of the proapoptotic BAX protein promotes the translocation of cytochrome c in rat embryo fibroblasts (Rosse et al., 1998); addition of BAX also induces the release of cytochrome c from isolated mitochondria (Jurgensmeier et al., 1998). These observations suggest a model in which the BCL-2 family proteins regulate the release of cytochrome c from the mitochondria during PCD, perhaps by their ability to form channels in the mitochondrial membrane. Once released, cytochrome c then binds Apaf-1 and procaspase-9 and, in the presence of dATP, promotes the activation of caspases and cell death. Thus, several questions about the apoptosis-promoting function of cytochrome c in intact cells remain unanswered. Is the translocation of cytochrome c important in all models of PCD? Is BAX required for the translocation of cytochrome c? Is cytochrome c translocation alone sufficient to activate caspases and promote cell death?

* To whom correspondence should be addressed (e-mail: ejohnson@pharmsun.wustl.edu).

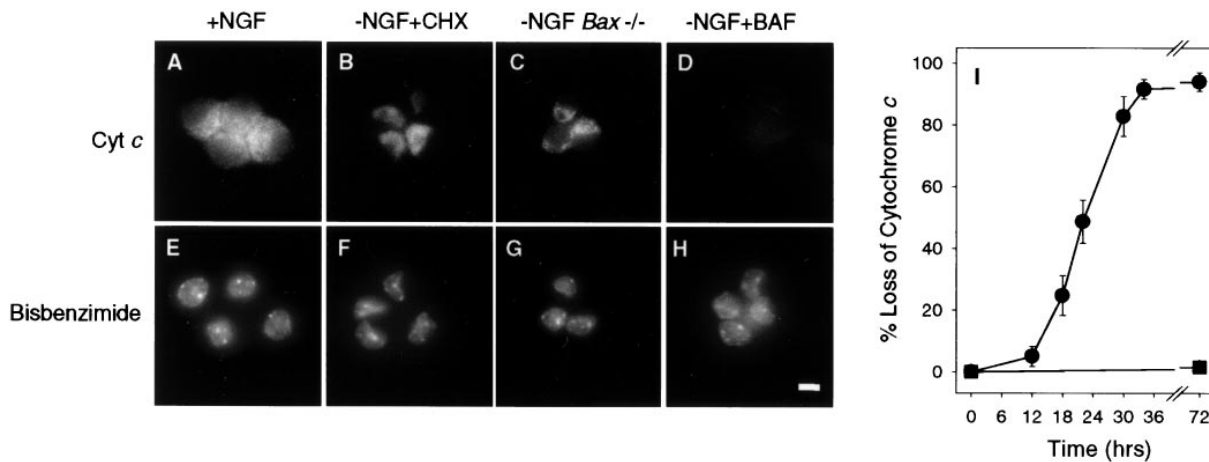


Figure 1. NGF Deprivation Induces Loss of Cytochrome c from the Mitochondria during Sympathetic Neuronal Death
Shown are representative photographs of anti-cytochrome c (cyt c)-immunostained (A–D) or the corresponding bisbenzimidate-stained nuclei (E–H) of sympathetic neurons from either wild-type or *Bax*-deficient mice. Neurons were maintained in vitro for 5 days in the presence of NGF and then treated for 72 hr as follows: wild-type neurons maintained in NGF (A and E); wild-type neurons deprived of NGF but in the presence of 1 μ g/ml cycloheximide (CHX) (B and F); *Bax*-deficient neurons deprived of NGF (C and G); and wild-type neurons deprived of NGF but in the presence of 50 μ M BAF (D and H). Scale bar, 10 μ m.

The time course of loss of punctate cytochrome c staining in sympathetic neurons deprived of NGF is shown (I). At various times after NGF deprivation, sympathetic neuronal cultures from wild-type mice were fixed and immunostained for cytochrome c, and the number of cells that had lost the punctate staining was determined (circles); these experiments were done in the presence of 50 μ M BAF to prevent any cell loss that otherwise would affect the quantitation. Also shown is the quantitation (for the 72 hr time point) of the lack of cytochrome c translocation seen in NGF-deprived, cycloheximide-saved sympathetic neurons (triangles) or the NGF-deprived sympathetic neurons from *Bax*-deficient mice (squares); data points corresponding to the triangles and squares are overlapping. Results are mean (\pm SEM) for at least three experiments with approximately 100 cells counted for each time point.

In this paper we have examined these questions by using the model of sympathetic neuronal death. Sympathetic neurons deprived of nerve growth factor (NGF) initiate PCD and undergo an apoptotic death within 24–48 hr after NGF removal in culture (Martin et al., 1988; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994; Deshmukh and Johnson, 1997). The NGF deprivation-induced sympathetic neuronal death is dependent on macromolecular synthesis (Martin et al., 1988) and BAX function (Deckwerth et al., 1996) and is prevented by caspase inhibitors such as boc-aspartyl(OMe)-fluoromethylketone (BAF) (Deshmukh et al., 1996; Troy et al., 1996; McCarthy et al., 1997). We found that mitochondrial localization of cytochrome c was lost during sympathetic neuronal death, an observation that is consistent with its translocation to the cytoplasm. This loss of cytochrome c from the mitochondria was dependent on events requiring macromolecular synthesis and BAX function. However, the cytoplasmic accumulation of cytochrome c per se was insufficient to activate caspases and cause cell death, since cytoplasmic microinjection of cytochrome c did not induce cell death in NGF-maintained sympathetic neurons. In contrast, microinjection of cytochrome c induced rapid cell death in NGF-deprived, *Bax*-deficient neurons or NGF-deprived, cycloheximide-treated neurons. Further studies indicated that sympathetic neurons needed to be deprived of NGF for 15–20 hr before they were able to undergo cell death after microinjection of cytochrome c.

Our data provide evidence that two parallel pathways are operative during NGF deprivation-induced sympathetic neuronal death. The first process, consistent with the model, is a macromolecular synthesis-dependent,

BAX-dependent release of cytochrome c. The second process is a macromolecular synthesis-independent, BAX-independent development of competence-to-die. Both processes are required to effect PCD in neurons after trophic factor deprivation.

Results

Loss of Cytochrome c from the Mitochondria Occurs before Caspase Function during Sympathetic Neuronal Death and Requires Protein Synthesis-Dependent Events and BAX Activity

Several biochemical and molecular events that occur after NGF deprivation in sympathetic neurons have been identified and sequentially ordered with respect to the point of BAX or caspase function. The translocation of cytochrome c from mitochondria is an event that occurs prior to the activation of caspases in several nonneuronal models of PCD. We examined whether the translocation of cytochrome c also occurred during neuronal PCD in NGF-deprived sympathetic neurons treated with the caspase inhibitor BAF; BAF addition prevents any cell death after NGF deprivation that would otherwise complicate quantitation of results caused by the loss of neurons from the population. In NGF-maintained sympathetic neurons, cytochrome c exhibited a punctate staining pattern, consistent with its expected mitochondrial localization (Figure 1A). The pattern of cytochrome c staining was dramatically altered in NGF-deprived, BAF-saved sympathetic neurons from a punctate mitochondrial pattern to a diffuse cytoplasmic pattern (Figure 1D). Analysis of the time course of this phenomenon showed

very little change in the intracellular localization of cytochrome c within the first 12 hr after NGF deprivation. However, by 22 hr after NGF deprivation, 50% of neurons showed diffuse cytochrome c staining, and greater than 90% had lost their mitochondrial cytochrome c staining by 36 hr after NGF deprivation (Figure 1).

Sympathetic neurons from *Bax*-deficient mice do not undergo apoptosis after NGF deprivation (Deckwerth et al., 1996). We examined whether BAX was required for the translocation of cytochrome c subsequent to NGF deprivation. Sympathetic neurons from *Bax*-deficient mice showed no change in cytochrome c localization and maintained their punctate cytochrome c staining pattern even after 72 hr of NGF deprivation (Figures 1C and 1I).

Macromolecular synthesis inhibitors such as cycloheximide prevent sympathetic neurons from undergoing PCD after NGF deprivation (Martin et al., 1988). To determine whether de novo protein synthesis was required for the translocation of cytochrome c during sympathetic neuronal death, we examined whether cycloheximide blocked the neuronal PCD pathway before or after the loss of cytochrome c from mitochondria. NGF-deprived, cycloheximide-saved neurons showed no change in cytochrome c localization and maintained their punctate cytochrome c staining pattern even after 72 hr of NGF deprivation (Figures 1B and 1I). The punctate staining pattern of cytochrome c seen in Figure 1 appeared more condensed in NGF-deprived, *Bax*-deficient sympathetic neurons and the NGF-deprived, cycloheximide-saved neurons as compared to the NGF-maintained neurons. This is presumably because NGF deprivation induces neuronal atrophy, thereby causing the cytosol to become asymmetrically distributed in the cell.

Thus, NGF deprivation induced the loss of cytochrome c from the mitochondria in sympathetic neurons. This event was dependent on macromolecular synthesis and BAX function but occurred prior to caspase activation, during sympathetic neuronal death.

Rescue with NGF Readdition Prevents Further Release of Cytochrome c during Neuronal Death

Sympathetic neurons deprived of NGF for 12 hr can be fully rescued upon readdition of NGF (Edwards et al., 1991). Thereafter, the NGF-deprived sympathetic neurons start becoming irreversibly committed to die; 50% of sympathetic neurons can no longer be rescued with NGF readdition by 22 hr after NGF deprivation, and virtually none can be rescued by 48 hr after NGF deprivation (Deckwerth and Johnson, 1993). Interestingly, the time courses with which sympathetic neurons are rescued with NGF readdition or with addition of the caspase inhibitor BAF are indistinguishable (Deshmukh et al., 1996). Therefore, the target of NGF rescue has been proposed to be either activated caspases themselves (similar to the target of BAF) or some event just proximal to the activation of caspases (Deshmukh et al., 1996).

As shown above, the translocation of cytochrome c was an event that immediately preceded caspase activation during sympathetic neuronal death. We examined whether rescue with NGF readdition blocked PCD before or after the event of cytochrome c translocation.

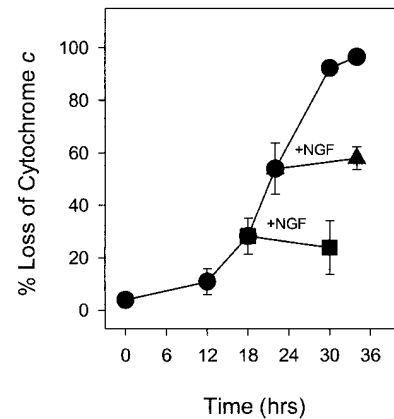


Figure 2. NGF Addition Prevents the Loss of Mitochondrial Cytochrome c during Sympathetic Neuronal Death

Cultures of mouse sympathetic neurons were deprived of NGF in the presence of 50 μ M caspase inhibitor BAF. At 0, 12, 18, 22, 30, or 34 hr after NGF deprivation, cultures were fixed and immunostained with cytochrome c antibodies, and the number of cells that had lost the punctate staining was quantitated (circles). In parallel studies, cultures of sympathetic neurons that were deprived of NGF in the presence of BAF for 18 hr (squares) or 22 hr (triangles) were washed and rescued with readdition of NGF (300 ng/ml). Twelve hours after NGF readdition, the rescued cells were fixed and immunostained with cytochrome c antibodies, and the number of cells that had lost the punctate staining pattern was determined. Results are mean (\pm SEM) for three experiments with approximately 100 cells counted for each time point.

Sympathetic neurons were deprived of NGF in the presence of BAF; BAF addition did not prevent cytochrome c translocation (as shown in Figure 1) but blocks any cell deaths that may otherwise affect the quantitation (Deshmukh et al., 1996). At 18 or 22 hr after NGF deprivation, NGF was added back to the cultures and the number of cells with diffuse cytochrome c staining 12 hr later was determined. Rescue with readdition of NGF completely prevented any further loss of cytochrome c from the mitochondria of NGF-deprived sympathetic neurons (Figure 2). The dramatic ability of NGF to abort the loss of cytochrome c contrasts with the inability of BAF to prevent cytochrome c translocation. These results indicate that whereas BAF targets caspases, NGF acts at some proximal step that is required for the loss of cytochrome c from mitochondria.

Microinjection of Cytochrome c Does Not Induce Cell Death in NGF-Maintained Sympathetic Neurons but Does in NGF-Deprived, *Bax*-Deficient Neurons and NGF-Deprived, Cycloheximide-Treated Neurons

Having demonstrated that loss of cytochrome c from the mitochondria occurred during NGF deprivation-induced sympathetic neuronal death, we next examined whether the cytoplasmic accumulation of cytochrome c was sufficient to induce cell death in sympathetic neurons. The binding of cytochrome c to Apaf-1 and procaspase-9 (in the presence of dATP) is sufficient to activate caspases and cause apoptotic changes in vitro (Li et al., 1997b). If all components of the caspase-activating machinery except cytochrome c are normally present in the cytosol, then the accumulation of cytochrome c in the

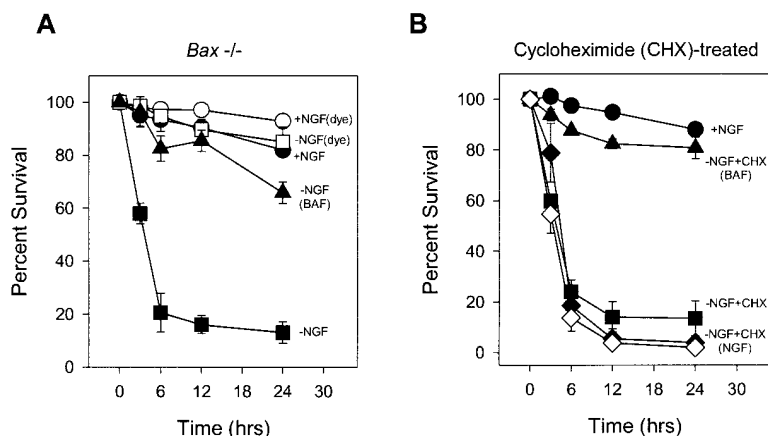


Figure 3. Microinjection of Cytochrome c Did Not Induce Cell Death in NGF-Maintained Sympathetic Neurons, but It Did in NGF-Deprived, *Bax*-Deficient or NGF-Deprived, Cycloheximide-Treated Neurons

(A) Sympathetic neurons from wild-type (circles) or *Bax*-deficient (squares and triangles) mice were grown in NGF for 4 days, then maintained for 24–48 hr either in the presence (circles) or absence (squares and triangles) of NGF. Cells were then injected with 25 mg/ml mammalian cytochrome c (filled symbols) or rhodamine dextran dye alone (open symbols). BAF (50 μ M) was added to one set of NGF-deprived cultures immediately after the microinjections (triangles). At each time point after the injections, the number of microinjected cells that remained viable was determined and expressed as a percentage of the total number of microinjected cells.

(B) Sympathetic neurons from wild-type mice were maintained in NGF for 4 days then treated for 24–48 hr with either NGF (circles), or anti-NGF plus 1 μ g/ml cycloheximide (CHX; squares, triangles, diamonds). Cells were microinjected with 25 mg/ml mammalian cytochrome c, and the number of viable cells remaining was determined as described in (A). Some NGF-deprived, cycloheximide-treated cultures were treated with 50 μ M BAF immediately after the microinjections (triangles), or with 300 ng/ml NGF either immediately after the microinjections (filled diamonds) or 30 min prior to the microinjections (open diamonds). Results are mean (\pm SEM) for three experiments with approximately 100 cells counted for each time point.

cytoplasm should be sufficient to activate caspases and cause cell death in sympathetic neurons. Therefore, we tested whether cytoplasmic microinjection of cytochrome c induced cell death in NGF-maintained sympathetic neurons. Microinjection of mammalian cytochrome c (25 mg/ml) did not induce cell death in NGF-maintained sympathetic neurons (Figure 3A; filled circles). The concentration of cytochrome c injected in this experiment is unlikely to be limiting because a 5-fold lower concentration of cytochrome c was sufficient to induce rapid cell death in these neurons under other conditions (see below).

The above data show that cytochrome c was insufficient to induce cell death in NGF-maintained sympathetic neurons. However, as shown in Figure 11, cytochrome c release that presumably leads to caspase activation is observed in neurons that had been deprived of NGF for at least 12 hr. Therefore, we examined whether sympathetic neurons needed to be deprived of NGF before microinjection of cytochrome c was able to kill neurons. We used two situations in which cells could be deprived of NGF yet remained alive: *Bax*-deficient or cycloheximide-treated sympathetic neurons. *Bax*-deficient sympathetic neurons do not undergo apoptosis when deprived of NGF because the cell death program is arrested at a BAX-dependent point prior to cytochrome c release (see Figure 1). These neurons remain viable in this NGF-deprived state for several weeks in culture (Deckwerth et al., 1996). Sympathetic neurons from *Bax*-deficient mice were deprived of NGF for 24–48 hr and then microinjected with cytochrome c. Microinjection of cytochrome c (25 mg/ml) rapidly induced cell death in NGF-deprived, *Bax*-deficient neurons (Figure 3A; filled squares). Greater than 40% of cells were dead by 3 hr after cytochrome c injection, and almost 80% were dead by 6 hr after the injections. Microinjection of dye alone did not induce cell death in these neurons (Figure 3A; open circles and open squares). Cell death induced by microinjection of cytochrome c was caspase

dependent, since PCD was blocked by the caspase inhibitor, BAF (Figure 3A; filled triangles).

Like sympathetic neurons from *Bax*-deficient mice, cycloheximide-treated neurons can also be deprived of NGF and be kept alive because these cells are arrested at a macromolecular synthesis-dependent step during neuronal death (Martin et al., 1988). We examined whether microinjection of cytochrome c induced cell death in cycloheximide-treated sympathetic neurons that were deprived of NGF for 24–48 hr. Microinjection of cytochrome c (25 mg/ml) rapidly induced cell death in the NGF-deprived, cycloheximide-treated neurons with 80% neurons dying within 6 hr after cytochrome c microinjection (Figure 3B; filled squares); dying cells showed chromatin condensation typically seen during apoptosis (data not shown). The cytochrome c-induced death of NGF-deprived, cycloheximide-treated neurons was also dependent on caspase activation, since PCD was prevented by the caspase inhibitor, BAF (Figure 3B; filled triangles). As described above, microinjection of cytochrome c did not induce cell death in NGF-maintained sympathetic neurons (Figure 3B; filled circles). Also, microinjection of dye alone did not induce cell death in NGF-deprived, cycloheximide-treated neurons (data not shown). Furthermore, microinjection of cytochrome c did not induce cell death in NGF-maintained sympathetic neurons treated with cycloheximide, indicating that cycloheximide treatment alone did not make sympathetic neurons sensitive to microinjection of cytochrome c (Figure 4A; filled triangles).

Thus, the cytoplasmic accumulation of cytochrome c was not sufficient to induce cell death in NGF-maintained sympathetic neurons. However, microinjection of cytochrome c induced a rapid, caspase-dependent death in cells that were maintained in the context of NGF deprivation, such as the NGF-deprived, *Bax*-deficient neurons or the NGF-deprived, cycloheximide-treated neurons.

Previous reports indicate that yeast (*Saccharomyces*

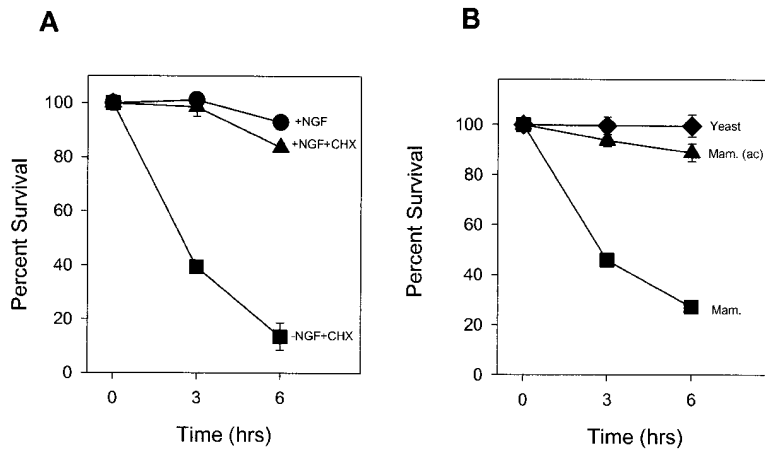


Figure 4. Cycloheximide Treatment Alone Is Not Sufficient to Make Sympathetic Neurons Susceptible to Microinjection of Mammalian Cytochrome c

(A) Sympathetic neurons from wild-type mice were maintained in NGF for 4 days then treated for 24–48 hr with either NGF (circles), NGF plus 1 μ g/ml cycloheximide (triangles), or anti-NGF plus 1 μ g/ml cycloheximide (squares). Cells were microinjected with 25 mg/ml mammalian cytochrome c, and the number of viable cells remaining was determined as described in the legend to Figure 3A.

(B) Specificity of cytochrome c in inducing sympathetic neuronal death. Sympathetic neurons from wild-type mice were deprived of NGF in the presence of cycloheximide for 24–48 hr and then injected with 25 mg/ml of either mammalian cytochrome c (squares),

partially acetylated mammalian cytochrome c (triangles), or yeast cytochrome c (diamonds). Cell survival was determined as described in the legend to Figure 3A.

Results are mean (\pm SEM) for three experiments with approximately 100 cells counted for each time point.

cerevisiae) cytochrome c or partially acetylated mammalian cytochrome c does not substitute for mammalian cytochrome c in activating caspases in cell-free extracts (Ellerby et al., 1997; Kluck et al., 1997b). Consistent with these in vitro results, we found that microinjection of yeast cytochrome c or partially acetylated mammalian cytochrome c did not induce cell death in NGF-deprived, cycloheximide-treated sympathetic neurons (Figure 4B).

Cytochrome c-Induced Death Is Dose Dependent

The experiments described above demonstrate that microinjection of 25 mg/ml cytochrome c induced cell death in NGF-deprived sympathetic neurons. This concentration of cytochrome c was chosen for the initial experiments because similar concentrations of cytochrome c are sufficient to induce cell death in human embryonic kidney 293 cells (Li et al., 1997a). To determine the minimum concentration of cytochrome c needed to induce cell death in sympathetic neurons, we examined the dose response for cytochrome c. Sympathetic neurons that were deprived of NGF for 24 hr in the presence of cycloheximide were microinjected with increasing concentrations of cytochrome c (0–25 mg/ml), and the extent of cell death was determined at various times after microinjection. Microinjection of cytochrome c showed an exponential dose response between 1 and 5 mg/ml with the maximum death-inducing response observed at 5 mg/ml cytochrome c (Figures 5A and 5B). Microinjection of 10 mg/ml or 25 mg/ml cytochrome c induced cell death with a time course that was identical to that observed with microinjection of 5 mg/ml of cytochrome c.

Cytochrome c-Induced Sympathetic Neuronal Death Is Prevented with the Caspase Inhibitor BAF but Not by Addition of NGF

One reason why microinjection of cytochrome c may not be able to induce cell death in NGF-maintained sympathetic neurons is that NGF exerts a block on the neuronal PCD pathway after the translocation of cytochrome c. For example, in addition to blocking the translocation of cytochrome c (as shown in Figure 2),

NGF might induce the phosphorylation of some proteins and directly prevent caspase activation. If this hypothesis were correct, addition of NGF to the NGF-deprived, cycloheximide-treated neurons at the time of cytochrome c microinjection should block cell death induced by cytochrome c in those neurons. To examine this possibility, NGF-deprived, cycloheximide-treated neurons were microinjected with 25 mg/ml cytochrome c. Immediately after the microinjections, the cultures were washed extensively and NGF was added to the medium. However, NGF addition did not prevent cell death induced by microinjection of cytochrome c (Figure 3B; filled diamonds). Similarly, NGF addition even 30 min prior to microinjection of cytochrome c, to allow sufficient time for any NGF signaling mechanisms to become established (Halegoua and Patrick, 1980), had no effect on cytochrome c-induced cell death (Figure 3B).

Although these results show that NGF was unable to prevent cell death after microinjection of cytochrome c, it remained possible that the concentration of cytochrome c microinjected into these neurons (25 mg/ml) was much greater than that required to activate caspases. The high concentration of cytochrome c may have overwhelmed any ability of NGF to block cell death physiologically after cytochrome c microinjection. As shown above, 2.5 mg/ml and 5 mg/ml cytochrome c represent the minimum concentrations of cytochrome c needed to induce cell death in sympathetic neurons (Figures 5A and 5B). We examined whether NGF addition could prevent cell death induced by microinjection of these threshold concentrations of cytochrome c. However, addition of NGF (30 min prior to injection) to NGF-deprived, cycloheximide-treated cultures microinjected with either 2.5 mg/ml or 5 mg/ml cytochrome c still had no effect on cell death induced by cytochrome c in these neurons (Figure 5C).

The inability of NGF to prevent cell death after microinjection of cytochrome c contrasts with the ability of the caspase inhibitor BAF to block cell death when added immediately after the microinjection (as shown in Figure 3). These results suggest that BAF, but not NGF, can prevent neuronal death once cytochrome c has translocated to the cytoplasm.

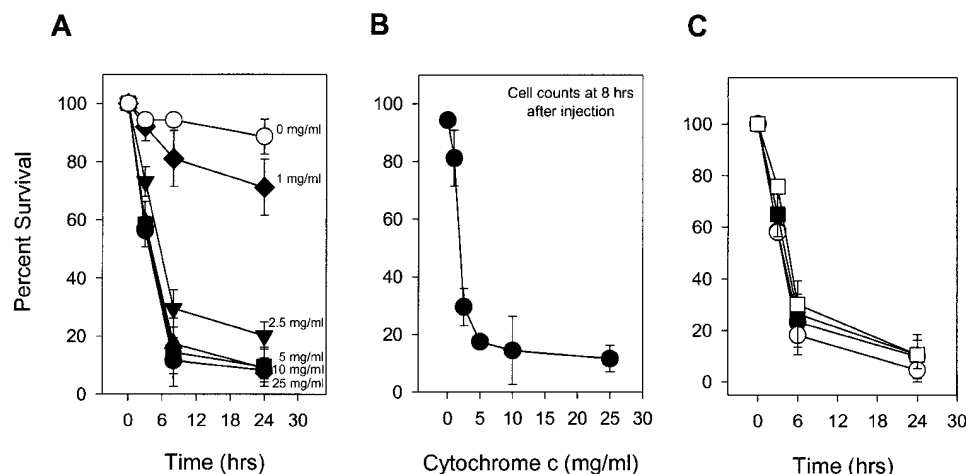


Figure 5. Killing by Cytochrome c Is Dose Dependent and Is Not Blocked by Acute Exposure to NGF

(A) Microinjection of cytochrome c induces sympathetic neuronal death in a dose-dependent manner. Sympathetic neurons were deprived of NGF in the presence of cycloheximide for 24–48 hr, then microinjected with rhodamine dextran dye alone (open circles) or 1 mg/ml (diamonds), 2.5 mg/ml (down triangles), 5 mg/ml (up triangles), 10 mg/ml (squares), or 25 mg/ml (closed circles) of mammalian cytochrome c. Neuronal survival at 0, 3, 8, or 24 hr after microinjection of cytochrome c was determined.

(B) Survival of sympathetic neurons at 8 hr postinjection (data from [A]) was plotted as a function of cytochrome c concentration.

(C) Addition of NGF does not block the sympathetic neuronal death induced by microinjection of cytochrome c. Sympathetic neurons were deprived of NGF in the presence of cycloheximide for 24–48 hr, then microinjected with 5 mg/ml (circles) or 2.5 mg/ml (squares) of mammalian cytochrome c. To a subset of these cultures, 300 ng/ml NGF was added 30 min prior to the microinjections (open symbols). Neuronal survival after the microinjections was determined as described above in Figure 3A.

Results are mean (\pm SEM) for three experiments with approximately 100 cells counted for each time point.

NGF Deprivation Is Required for Sympathetic Neurons to Develop Competence-to-Die with Microinjection of Cytochrome c

The results described above indicate that microinjection of cytochrome c was insufficient to induce cell death in NGF-maintained sympathetic neurons, whereas similar microinjections rapidly induced cell death in sympathetic neurons that were maintained in the context of NGF deprivation such as the NGF-deprived, *Bax*-deficient neurons or NGF-deprived, cycloheximide-treated neurons. Our results argue against the possibility that NGF-maintained neurons are resistant to cell death induced by microinjection of cytochrome c, because NGF blocks neuronal PCD after the cytoplasmic accumulation of cytochrome c. Therefore, we considered the possibility that NGF deprivation per se induces other events that are required for sympathetic neurons to become sensitive to cytochrome c-induced death.

We examined whether cytochrome c could induce cell death in sympathetic neurons if these neurons were deprived of NGF immediately after microinjection or if sympathetic neurons need to be deprived of NGF for a period of time before they developed competence-to-die in response to microinjection of cytochrome c. Microinjection of cytochrome c did not induce cell death in sympathetic neurons that were deprived of NGF immediately after the injection (Figure 6A; filled triangles). In contrast, cytochrome c induced rapid cell death in sympathetic neurons that were deprived of NGF for 24 hr prior to the injection (described in previous sections and Figure 6A; filled squares). NGF was withdrawn in the presence of cycloheximide to prevent any cell death that would be caused by NGF deprivation alone, as described previously.

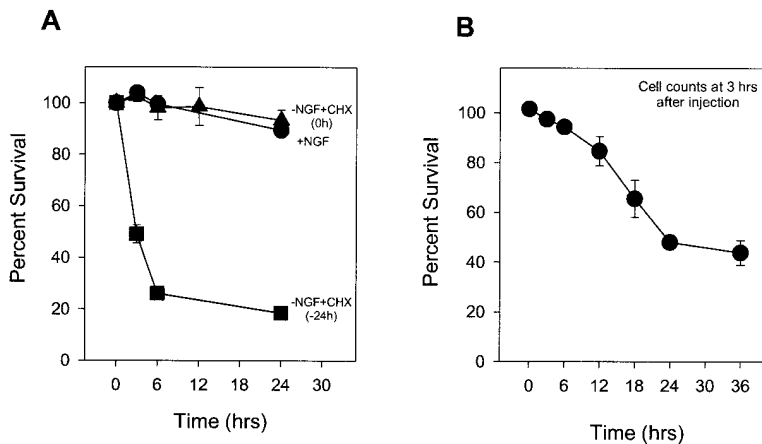
To determine how long sympathetic neurons must be deprived of NGF before they developed competence-to-die with microinjection of cytochrome c, sympathetic neurons were deprived of NGF in the presence of cycloheximide. At various times after NGF deprivation, neurons were microinjected with 5 mg/ml cytochrome c, and the extent of cell death was determined 3 hr after the injections. The 3 hr time point was chosen because it was the minimum time period needed to detect substantial cell death. Sympathetic neurons became competent-to-die with microinjection of cytochrome c by 24 hr after NGF deprivation; NGF deprivation for longer periods did not result in any further increase in cell death (Figure 6B). Half of the sympathetic neurons developed competence-to-die by 16–18 hr after NGF deprivation.

These results indicate that NGF deprivation was required to induce a state of competence in sympathetic neurons that allows them to undergo cell death with microinjection of cytochrome c. Since sympathetic neurons develop this competence in the presence of cycloheximide (NGF-deprived, cycloheximide-treated neurons) or in the absence of BAX (NGF-deprived, *Bax*-deficient neurons), the development of competence does not require protein synthesis or BAX function.

Discussion

Loss of Cytochrome c from the Mitochondria during Sympathetic Neuronal Death

In this paper, we examined how caspases become activated during neuronal death. We asked whether mitochondrial release of cytochrome c, which occurs in several nonneuronal models of apoptosis, also occurs



18, 24, or 36 hr after NGF deprivation, neurons were microinjected with 5 mg/ml mammalian cytochrome c, and the number of viable neurons remaining at 3 hr after the microinjection of cytochrome c was determined. Results are mean (\pm SEM) for three experiments with approximately 100 cells counted for each time point.

during sympathetic neuronal death. We found that cytochrome c is lost from the mitochondria after NGF deprivation during sympathetic neuronal death. Consistent with observations in other models of PCD, loss of cytochrome c from the mitochondria occurs prior to caspase function during neuronal death (Figure 1).

Several recent reports suggest that the BCL-2 family of proteins, BAX in particular, regulates the release of cytochrome c during apoptosis (Reed, 1997b). Sympathetic neurons are completely dependent on BAX for undergoing apoptosis (Deckwerth et al., 1996). Therefore, we tested whether BAX was required for the release of cytochrome c during apoptosis. Our results show that NGF-deprived sympathetic neurons from *Bax*-deficient mice maintained their punctate staining pattern of cytochrome c, indicating that BAX was required for the loss of cytochrome c from mitochondria during neuronal apoptosis (Figure 1). Whether the release of cytochrome c occurred as a consequence of the insertion of BAX into the mitochondria of sympathetic neurons and formation of ion-selective channels is not known.

Our results support the hypothesis that the BAX-dependent release of cytochrome c is important in mediating caspase activation during trophic factor deprivation-induced neuronal death. The inability of cytochrome c to translocate after NGF deprivation in *Bax*-deficient sympathetic neurons correlated with the inability of these neurons to undergo NGF deprivation-induced apoptosis. Furthermore, microinjection of cytochrome c into the cytoplasm of NGF-deprived, *Bax*-deficient sympathetic neurons, which bypasses the cytochrome c translocation defect observed in these neurons, allowed these cells to undergo a rapid, caspase-dependent cell death (Figure 3). Since microinjection of cytochrome c alone was sufficient to complement the apoptotic defect in the NGF-deprived, *Bax*-deficient sympathetic neurons, the essential function of BAX in neuronal apoptosis must likely be to mediate the translocation of cytochrome c. Therefore, these results imply that the translocation of cytochrome c is important for mediating NGF deprivation-induced sympathetic neuronal death. Experiments that directly examine the requirement for cytochrome c in neuronal apoptosis must

await the generation of conditional null mutants of cytochrome c that eliminate the death-promoting activity but maintain its essential function in oxidative phosphorylation.

Inhibitors of macromolecular synthesis, such as cycloheximide, prevent the NGF deprivation-induced apoptosis of sympathetic neurons (Martin et al., 1988). We found that addition of cycloheximide to NGF-deprived sympathetic neurons blocked apoptosis prior to the loss of cytochrome c from the mitochondria (Figure 1). Thus, a macromolecular synthesis-dependent event(s) was required for the release of cytochrome c from mitochondria after NGF deprivation. Taken together, these results demonstrate that NGF deprivation induced a macromolecular synthesis-dependent, BAX-dependent loss of cytochrome c from the mitochondria during neuronal PCD.

NGF Readdition Rescues Sympathetic Neurons up to the Point of Cytochrome c Release during PCD

PCD induced by NGF deprivation in sympathetic neurons can be aborted by the readdition of NGF even if NGF is added very late during the death process (Edwards et al., 1991; Deckwerth and Johnson, 1993). Although NGF signals postrationally to block neuronal PCD, the mechanism by which NGF readdition acts to prevent neuronal apoptosis is not known. Previous studies could not resolve any temporal difference between the time course of rescue with addition of NGF or BAF. Hence, the target of NGF rescue had been proposed to be either activated caspases or some event immediately preceding caspase activation (Deshmukh et al., 1996).

In this study, we have identified important differences between the mechanisms of NGF and BAF rescue. We find that NGF readdition prevented the loss of cytochrome c from mitochondria during sympathetic neuronal death (Figure 2), whereas BAF addition did not (Figures 1 and 2). Furthermore, our results indicate that NGF was unable to prevent neuronal apoptosis once cytochrome c accumulated in the cytoplasm, since NGF readdition did not prevent cell death induced by microinjection of cytochrome c, whereas BAF did (Figures 3B

Figure 6. NGF Deprivation Is Needed for Sympathetic Neurons to Develop Competence-to-Die after Microinjection of Cytochrome c

(A) Sympathetic neurons were grown in NGF for 4 days and then were maintained in the presence of NGF for 2 days (circles and triangles) or deprived of NGF in the presence of cycloheximide for 24 hr (squares). Neurons were then microinjected with 5 mg/ml mammalian cytochrome c, and cell survival was determined as described above in Figure 3A. A subset of NGF-maintained cultures was deprived of NGF in the presence of cycloheximide immediately after the microinjections (triangles).

(B) Sympathetic neurons were maintained in NGF for 4 days and then deprived of NGF in the presence of cycloheximide. At 0, 3, 6, 12,

and 5C). These results are consistent with a model in which NGF can block the neuronal PCD pathway only until the point of cytochrome c release. This is further supported by the observation that the time course of loss of cytochrome c (Figure 11) is identical to the time course with which NGF loses its ability to prevent neuronal death (Deckwerth and Johnson, 1993).

Sympathetic Neurons Must Develop Competence before They Become Susceptible to Cytochrome c-Induced Cell Death

Although several reports have recently demonstrated that cytochrome c translocates from the mitochondria to the cytosol during PCD, few have addressed whether the cytoplasmic accumulation of cytochrome c is sufficient to activate caspases and cause apoptosis in cells (Liu et al., 1996; Du et al., 1997; Kharbanda et al., 1997; Kluck et al., 1997a; Li et al., 1997a; Srinivasan et al., 1998). Our results show that microinjection of cytochrome c was not sufficient to activate cell death in sympathetic neurons maintained in NGF. However, microinjection of cytochrome c induced rapid cell death in sympathetic neurons that were maintained in the absence of NGF, such as the *Bax*-deficient and cycloheximide-treated sympathetic neurons (Figure 3). Death induced by microinjection of cytochrome c in sympathetic neurons occurred as a consequence of caspase activation, since PCD was completely inhibited by the caspase inhibitor BAF (Figures 3A and 3B). This result argues against the possibility that cytochrome c was merely toxic to the cells. Furthermore, the inability of yeast cytochrome c or partially acetylated mammalian cytochrome c to induce cell death in sympathetic neurons provides additional evidence for the specificity of the cell death induced by cytochrome c in these experiments (Figure 4B).

Previous reports suggest that cytochrome c-induced death requires the expression of caspase-3 (Li et al., 1997a). MCF7F cells lack caspase-3 and, as a consequence, are resistant to cytochrome c-induced cell death. However, unlike MCF7F cells, sympathetic neurons express caspase-3 (Deshmukh et al., 1996). Hence, the inability of cytochrome c to induce cell death in NGF-maintained sympathetic neurons is not readily explained by any obvious deficiency of caspase-3 in these neurons.

So why does cytochrome c induce sympathetic neuronal death in the absence, but not the presence, of NGF? Our data indicate that NGF deprivation is needed for sympathetic neurons to develop competence-to-die with microinjection of cytochrome c. First, microinjection of cytochrome c induced rapid cell death in NGF-deprived, *Bax*-deficient neurons and NGF-deprived, cycloheximide-treated sympathetic neurons, whereas even a 5-fold greater concentration of cytochrome c did not induce any cell death in NGF-maintained sympathetic neurons (Figures 3, 4, and 5). Second, the susceptibility of NGF-deprived, *Bax*-deficient or NGF-deprived, cycloheximide-treated neurons to cytochrome c-induced death was not caused solely by their decreased metabolic rates (compared to the NGF-maintained neurons). Decreasing the metabolic rate in NGF-maintained sympathetic neurons by addition of cycloheximide, deprivation of glucose, or both treatments combined did not

make these neurons susceptible to cytochrome c-induced death (Figure 4A and data not shown). Third, the absence of NGF, and not cycloheximide treatment or *Bax* deficiency per se, induced susceptibility to cytochrome c-induced death. As mentioned above, microinjection of cytochrome c did not induce cell death in NGF-maintained sympathetic neurons treated with cycloheximide; similarly, microinjection of cytochrome c did not induce cell death in *Bax*-deficient sympathetic neurons maintained in NGF (data not shown).

We considered the possibility that one reason NGF deprivation is needed for sympathetic neurons to become susceptible to microinjection of cytochrome c was that NGF may signal posttranslationally to block the cytochrome c-induced activation of caspases and, thus, block cell death. Our data argue against this possibility. Addition of NGF to NGF-deprived, cycloheximide-treated neurons did not block the cell death induced by microinjection of cytochrome c even when NGF was added 30 min prior to the injections to allow time for any posttranslational modifications to occur (Figure 5C). Conversely, removal of NGF from NGF-maintained sympathetic neurons a few hours prior to the injections did not make these neurons susceptible to cytochrome c-induced cell death (Figure 6).

These results indicate that sympathetic neurons needed to be deprived of NGF for a period before the neurons developed competence-to-die with microinjection of cytochrome c. Half the neurons developed competence-to-die by 16–18 hr after NGF deprivation, and all neurons did so by 24 hr after NGF deprivation (Figure 6). Thus, NGF deprivation induced a state of competence in sympathetic neurons that allowed these neurons to initiate a caspase-dependent death after microinjection of cytochrome c. Furthermore, the development of competence required neither macromolecular synthesis nor BAX function, since the NGF-deprived, *Bax*-deficient and NGF-deprived, cycloheximide-treated sympathetic neurons developed competence-to-die with microinjection of cytochrome c.

Based on these results, we propose that NGF deprivation-induced cell death in sympathetic neurons requires the cooperation of two events (summarized in Figure 7): first, a macromolecular synthesis-dependent, BAX-dependent release of cytochrome c from the mitochondria; second, the development of competence that does not require macromolecular synthesis or BAX function. Neither event by itself is sufficient to induce cell death. Since the time courses for the loss of cytochrome c (Figure 11) and the development of competence (Figure 6B) are similar, these two events most likely occur simultaneously, albeit independently, after NGF deprivation in sympathetic neurons.

The molecular basis for the development of competence is not known. One hypothesis is that the development of competence may involve either the sequestering or degradation of an antiapoptotic factor that blocks cell death after the translocation of cytochrome c. Recent reports indicate that antiapoptotic proteins such as BCL-X_L and the inhibitor-of-apoptosis proteins (IAPs) prevent apoptosis by acting at a point distal to the release of cytochrome c (Roy et al., 1997; Clem et al., 1998; Deveraux et al., 1998; Duckett et al., 1998; Hu et al.,

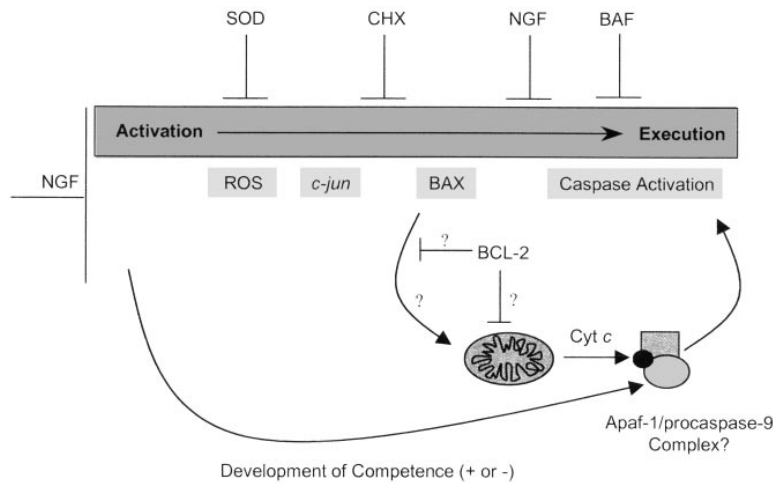


Figure 7. Sympathetic Neuronal Death Induced by NGF Deprivation

Sequence of events occurring after NGF deprivation in sympathetic neurons undergoing apoptosis. ROS, reactive oxygen species; SOD, superoxide dismutase; CHX, cycloheximide; cyt c, cytochrome c. Overexpression of SOD presumably inhibits sympathetic neuronal apoptosis by inhibiting the increase in ROS. The event inhibited by CHX is unknown. One possibility is that CHX may inhibit the expression of c-jun or a gene product whose expression is regulated by c-jun. The development of competence is shown as + or - to denote either the accumulation or degradation of a factor. The translocation of BAX to mitochondria is inferred from data in non-neuronal cell lines undergoing apoptosis. Two possible sites of BCL-2 action are suggested: inhibition of BAX translocation, or inhibition of the release of cytochrome c subsequent to BAX translocation. Question marks indicate uncertainty between these possibilities.

1998; Pan et al., 1998). Alternatively, the development of competence may involve the accumulation of a cofactor that is required for caspase activation. One such candidate is dATP. Addition of 1 mM dATP (or higher amounts of ATP) is required for cytochrome c to activate caspases in vitro (Liu et al., 1996). Whether dATP is the required cofactor needed for caspase activation in vivo remains unclear. We found that microinjection of dATP (25 mM dATP pipette concentration corresponding to an approximate final intracellular concentration of 0.25–2.5 mM dATP after injection) and cytochrome c together still did not induce cell death in NGF-maintained sympathetic neurons (data not shown). However, a different, as yet unknown, cofactor may be required for cytochrome c to activate caspases in sympathetic neurons.

Why Might Neurons Require the Development of Competence before Cytochrome c Can Induce Cell Death?

The observation that sympathetic neurons must be deprived of NGF before they developed competence-to-die with microinjection of cytochrome c contrasts with the recent report that microinjection of cytochrome c is sufficient to activate a caspase-dependent death in HeLa and 293 cells (Li et al., 1997a). Whether this difference in susceptibility to cytochrome c microinjection is reflective of a difference between a mitotic and a postmitotic cell is unclear. We note, however, that the decision to undergo apoptosis needs to be tightly regulated particularly in postmitotic cells, such as neurons, since these cells cannot be easily replaced. In contrast, the death of mitotic cells need not be catastrophic for the organism, since these cells can be replaced by normal cell proliferation. Several situations in the life span of neurons may result in accidental or physiological release of cytochrome c into the cytoplasm. For example, extrinsic or intrinsic perturbations may transiently depolarize mitochondria and result in some release of cytochrome c (Scarlett and Murphy, 1997; Yang and Cortopassi, 1998). Alternatively, cytochrome c release might

occur during normal mitochondrial turnover and biogenesis in neurons. Neurons, being irreplaceable, may, therefore, have evolved a mechanism that requires both cytochrome c release and the development of competence to prevent “accidental” activation of caspases in such situations. In contrast, physiological apoptotic stimuli, such as trophic factor deprivation, would induce both cytochrome c release and the development of competence, thereby ensuring the physiologically appropriate activation of caspases and cell death.

Experimental Procedures

Reagents

Bovine cytochrome c, partially acetylated horse cytochrome c, and yeast cytochrome c were purchased from Sigma (St. Louis, MO). All other reagents were also purchased from Sigma unless otherwise stated. Collagenase and trypsin were purchased from Worthington Biochemical (Freehold, NJ). Caspase inhibitor boc-aspartyl(OMe)-fluoromethylketone (BAF) was purchased from Enzyme Systems Products (Livermore, CA). Untimed-pregnant ICR mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Breeding and genotyping of *Bax*-deficient mice has been described previously (Knudson et al., 1995; Deckwerth et al., 1996).

Sympathetic Neuronal Cultures

Primary cultures of sympathetic neurons from the superior cervical ganglion (SCG) neurons were prepared from postnatal day-1 (P1) mice essentially as described previously for rats (Johnson and Argiro, 1983; Deshmukh et al., 1996). Briefly, the dissected ganglia were treated with collagenase (1 mg/ml), then trypsin (2.5 mg/ml) for 30 min each at 37°C. The ganglia were triturated, and the dissociated cells were plated on collagen-coated dishes in NGF-containing medium (AM50). This medium contained Eagle’s minimum essential medium with Earle’s salts (Life Technologies, Inc., Gaithersburg, MD) with the addition of 50 ng/ml 2.5 S NGF, 10% fetal calf serum, 2 mM glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin; 20 µM fluorodeoxyuridine, 20 µM uridine, and 3.3 µg/ml aphidicolin were also included to reduce the number of nonneuronal cells.

Culture Conditions

Sympathetic neuronal cultures were grown in NGF-containing medium (AM50) for 4–5 days and then either maintained in AM50 or treated with various conditions as follows. For NGF deprivation,

cultures were rinsed twice with medium lacking NGF (AM0: AM50 medium without NGF), followed by addition of AM0 containing goat anti-NGF neutralizing antibody (Ruit et al., 1990). For NGF deprivation in the presence of cycloheximide or the caspase inhibitor, 1 μ g/ml cycloheximide or 50 μ M BAF was added, respectively, to the anti-NGF-containing medium. For experiments in which NGF was readded to cultures after NGF deprivation, cultures were rinsed three times with the AM50 medium and then incubated in AM50 medium containing 300 ng/ml NGF (instead of 50 ng/ml); the greater concentration of NGF was added to ensure complete neutralization of the anti-NGF antibodies that may have remained from the previous treatments.

Immunohistochemistry

Neuronal cultures were immunostained as previously described (Easton et al., 1997). Briefly, sympathetic neurons (1500–3000 cells) were grown on collagen-coated, 2-well glass chamber slides (Nalge Nunc Int., Naperville, IL) in the appropriate medium. Cultures were washed once with phosphate-buffered saline (PBS) and then fixed with freshly made 4% paraformaldehyde in PBS for 30 min at 4°C. Cultures were then washed three times with Tris-buffered saline (TBS; 100 mM Tris-HCl [pH 7.6] and 0.9% NaCl), exposed to blocking solution (TBS containing 5% goat serum and 0.3% Triton X-100) for 30 min at room temperature (RT), and incubated in anti-cytochrome c primary antibody (Pharmingen, San Diego, CA) solution overnight at 4°C. The primary antibody was diluted 1:1000 (final concentration of 0.5 μ g/ml) in TBS containing 1% goat serum and 0.3% Triton X-100. Cultures were then washed three times with TBS and incubated in an anti-mouse FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) solution for 2–4 hr at 4°C. The secondary antibody was diluted 1:300 (final concentration of 2 μ g/ml) in TBS containing 1% goat serum and 0.3% Triton X-100. After the secondary antibody incubation, cultures were washed twice in TBS and stained with the nuclear dye bisbenzimidazole (Hoechst 33258 used at 1 μ g/ml; Molecular Probes Inc., Eugene, OR) for 15 min at RT. After washing twice again with TBS and adding one drop of mounting medium (50% glycerol, 0.1% paraphenylenediamine in PBS) on the cells, a glass coverslip was placed of the cells and the samples were examined by fluorescence microscopy.

Cell Counts for Loss of Cytochrome c

Mouse sympathetic neurons that had been maintained in culture for 4 days in NGF-containing medium were deprived of NGF in the presence of the caspase inhibitor BAF. At various times after NGF deprivation, cultures were fixed and immunostained with anti-cytochrome c antibodies. NGF-maintained sympathetic neurons exhibited a punctate staining pattern with anti-cytochrome c antibodies, and this staining pattern became very diffuse upon NGF deprivation. For each time point, the number of cells that had lost the punctate staining pattern for cytochrome c, from a random sampling of 100–150 cells, was determined by a blinded observer. These experiments were done in the presence of BAF to prevent any cell loss that would otherwise affect the quantitation.

For experiments in which we examined the effect of NGF addition on cytochrome c translocation, cultures were washed and treated with NGF readdition as described in Culture Conditions; the percentage of cells that had lost the mitochondrial cytochrome c staining was quantitated exactly as described above.

Microinjections and Quantitation of Cell Death

For microinjections, sympathetic neuronal cultures (1500–3000 cells), grown in the appropriate medium on collagen-coated, 35 mm dishes (Corning, Corning, NY), were switched to Leibovitz's L-15 medium (Life Technologies Inc., Gaithersburg, MD) containing 100 μ g/ml penicillin and 100 μ g/ml streptomycin just prior to injections. To identify the injected cells, the microinjection solution (100 mM KCl and 10 mM KP_i [pH 7.4]) contained rhodamine dextran (4 mg/ml). Microinjection solution containing rhodamine dextran (dye) alone or dye plus cytochrome c (various concentrations diluted in water and freshly prepared for each experiment) was injected into the cytoplasm of neurons by using Femtotips needles (Eppendorf Inc., Madison, WI). Immediately after the injections, the number of viable cells

injected was determined by counting the number of rhodamine-positive cells that had intact, phase-bright cell bodies. Cultures were then switched to the appropriate medium and, at various times after injections, the number of viable, injected neurons remaining was determined by using the same counting criterion. To assess whether the cytochrome c-injected cells exhibited condensed chromatin, cells were fixed with 4% paraformaldehyde (in PBS) 3 hr after cytochrome c injection and stained with the nuclear dye bisbenzimidazole.

An estimation of the amount of cytochrome c injected in these experiments is calculated as follows. SCG cell volume is calculated to be approximately 4 fl based on the average cell diameter of 20 μ m. We estimate that the amount injected into cells corresponds to 1%–10% of the total cell volume. Based on these calculations, injection of 25 mg/ml and 5 mg/ml pipette concentrations of cytochrome c are likely to represent final intracellular concentrations of 0.25–2.5 mg/ml (1–10 pg injected) and 0.05–0.5 mg/ml (0.2–2 pg injected) of cytochrome c, respectively. Likewise, injection of 25 mM dATP is likely to represent a final intracellular concentration of 0.25–2.5 mM dATP in cells.

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