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# Bax and caspases regulate increased production of mitochondria-derived reactive species in neuronal apoptosis: LACK of A role for depletion of cytochrome c from the mitochondrial electron transport chain

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ABSTRACT

A Bax-dependent increase of reactive oxygen species (ROS) and other reactive species (RS) occurs after withdrawing NGF from mouse sympathetic neurons in cell culture. Possible mechanisms underlying the increased ROS/RS are leakage of electrons from the mitochondrial electron transport chain secondary to caspase cleavage of respiratory complexes or leakage secondary to depletion of cytochrome *c* from the chain. We previously demonstrated that deletion of Bax or caspase 3 from these cells reduces ROS/RS production to near baseline levels indicating a central role for both Bax and caspase 3 in generating the ROS/RS. Here we depleted cytochrome *c* to a similar level in neurons from wild type and *bax* hemizygous or knockout mice by NGF withdrawal or treatment with $H_2O_2$. Death was prevented with a caspase inhibitor that caused a partial reduction of ROS/RS levels but did not completely prevent the ROS/RS increase. ROS/RS was highest in *bax* wild-type cells, lowest in *bax* knockout cells, and at an intermediate level in the *bax* hemizygous cells. These and our previous findings indicate that Bax and caspase 3 are necessary for the increased ROS/RS after withdrawing NGF from these cells and that little or none of the increased ROS/RS are secondary to a depletion of cytochrome *c* from the electron transport chain.



## 1. Introduction

At least half of the neurons generated during vertebrate neurogenesis die by apoptosis. Availability of a required neurotrophic factor is a primary determinant of which cells survive developmental death. Those neurons obtaining sufficient quantities of neurotrophin live while those that do not die. This process is involved in sculpting the developing nervous system [1]. The most extensively investigated model of developmental neuronal apoptosis consists of rat or mouse sympathetic neurons in cell culture deprived of nerve growth factor (NGF) [2–5]. As with many other types of neurons, the apoptotic death of these cells depends on the proapoptotic Bcl-2 family member, Bax [6]. Most Bax resides in the cytoplasm although some is associated with the outer mitochondrial membrane (OMM) of NGF-replete sympathetic neurons [7]. After NGF withdrawal, cytosolic Bax becomes tightly associated with the OMM where it permeabilizes the membrane and induces release of apoptogenic substances from the mitochondrial intermembrane space into the cytoplasm [8–10]. The most important of these factors for the death of sympathetic neurons and many other cell types is cytochrome *c*. Once in the cytoplasm, cytochrome *c* activates caspase-9 by inducing formation of the apoptosome [11]. Caspase-9 then activates downstream effector caspases that cleave many critical protein substrates, causing cell death.

The mechanism(s) by which Bax causes OMM permeabilization remains unclear [12,13]. Withdrawing NGF from rat or mouse sympathetic neurons causes increased levels of reactive oxygen species (ROS) and reactive species (RS) lying downstream of ROS in those cells [5,10,14–17]. These ROS are Bax-dependent and derive from the mitochondrial electron transport chain. A known mechanism by which Bax can increase cellular ROS is by caspase cleavage of respiratory complexes secondary to Bax-induced caspase activation and translocation to the mitochondrial

*Abbreviations:* NGF, nerve growth factor; ROS, reactive oxygen species; RS, reactive species; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; $\Delta\psi m$,, mitochondrial membrane potential; $O_2$.-, superoxide; CM-$H_2$ DCFDA, 5-(and-6)-chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate; $TMRM^+$, tetramethylrhodamine methyl ester; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; BAF, boc-aspartyl(OMe)-fluoromethylketone

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intermembrane space [18,19]. Consistent with this mechanism in mouse sympathetic neurons, we reported that a broad-spectrum caspase inhibitor inhibits increased production of ROS/RS in NGF-deprived cells and that caspase 3 deletion blocks almost all of it. Another mechanism by which Bax might increase cellular mitochondrial ROS production is by depleting the chain of cytochrome *c* [20,21]. Such depletion could, ostensibly, increase leakage of electrons to molecular oxygen to produce the free radical ROS, superoxide ($O_2^{\cdot -}$). Other cellular ROS and RS are then produced downstream of $O_2^{\cdot -}$. Antioxidants block cytochrome *c* release and apoptotic death in NGF-deprived sympathetic neurons and other cells while pro-oxidants cause cytochrome *c* redistribution and death [5,16,17]. A recent finding demonstrates that Bax can increase mitochondrial-derived ROS in HepG2 and H9c2 cells and that these ROS then increase association of Bax with mitochondria in a positive feedback cycle that causes release of cytochrome *c* from all mitochondria in a cell over a short period [22]. Our published reports suggest that a similar mechanism exists in NGF-deprived sympathetic neurons [10,16,17]. Therefore, ROS/RS appear to lie both up- and downstream of cytochrome *c* redistribution in NGF-deprived sympathetic neurons and other cells and are a critical component of the mechanism by which Bax causes cytochrome *c* release. Our published findings suggest that most of the pro-oxidant effect of Bax is mediated via activation of caspase 3. Here we present evidence that depletion of cytochrome *c* from the electron transport chain makes little if any contribution to the increased Bax-dependent ROS/RS following NGF withdrawal. Our findings also suggest that, under some circumstances, Bax may have additional pro-oxidant effects that are independent of caspase activity.

## 2. Material and methods

### 2.1. Materials

5-(and-6)-chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate (CM–$H_2$DCFDA) and tetramethylrhodamine methyl ester ($TMRM^+$) were obtained from Molecular Probes (Eugene, OR). NGF 2.5S was from Harlan Bioproducts (Indianapolis, IN). Unless otherwise stated all other reagents were from Sigma (St. Louis, MO).

### 2.2. Breeding and genotyping of mice

Knockout ($bax^{-/-}$), hemizygous ($bax^{+/-}$), and wild-type ($bax^{+/+}$) mice were generated by mating mice hemizygous for the *bax* allele (Jackson Labs, Bar Harbor, ME) [23]. Genomic DNA was prepared from the tail of each pup using a Wizard Prep kit (Promega, Madison, WI) and genotype determined by PCR as previously described [17].

### 2.3. Cell culture

Superior cervical ganglia were dissected from newborn mice. Neurons were then enzymatically and mechanically dissociated from the ganglia, plated on a collagen substrate on #1 glass coverslips, and maintained in 35 mm culture dishes [6,17]. These coverslips were placed in an Attofluor cell chamber (Molecular Probes) for microscopy. Separate cultures were established for the ganglia from each pup of $bax^{+/-}$ X $bax^{+/-}$ matings. Cells to be used for immunoblotting experiments were plated on 35 mm culture dishes. Cells for survival experiments were plated on 35 mm or 24-well culture dishes. Neurons from ½ to 1 ganglion were plated per culture for most experiments. Cultures were maintained in medium containing Eagle's minimum essential medium with Earle's salts w/o L-glutamine (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine, 20 μM uridine, 1.4 mM L-glutamine, and 50 ng/ml 2.5S NGF. Cultures were incubated at 35 °C in an incubator having a 5% $CO_2$ and 95% air atmosphere. Cultures were deprived of NGF by incubating them in culture medium containing a NGF-neutralizing antibody (Harlan Bioproducts or Abcam Inc., Cambridge, MA) and lacking NGF. All data presented are combined from experiments done with neurons from at least three separate platings. All experiments were begun when cultures were 6–9 days old.

### 2.4. Confocal microscopy

Confocal microscopy experiments were done with a Nikon C1 laser-scanning confocal microscope (Southern Micro Instruments, Marietta, GA) attached to a Nikon Eclipse TE300 inverted microscope. Capture of images was accomplished by EZC1 software. Neurons were scanned at 512 × 512 pixel resolution. Confocal pinhole size was always the same within an experiment.

### 2.5. ROS/RS and mitochondrial membrane potential ($\Delta\psi_m$) measurement

The ROS/RS-sensitive dye 5-(and-6)-chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate (CM-$H_2$ DCFDA) was used to determine neuronal ROS and other RS levels. This dye is readily membrane-permeant. Once in cells, it is effectively trapped by binding to cellular thiols. CM-$H_2$ DCFDA is only faintly fluorescent in reduced form but becomes intensely fluorescent when oxidized by several ROS and other RS [24]. We extensively characterized the use of this dye in sympathetic neurons and found it to be superior to other similar dyes for assessing changes in the ROS/RS levels in these cells [16,17]. Cultures were incubated for 20–25 min at 35°C in the appropriate experimental medium containing CM-$H_2$ DCFDA (10 μM). They were then washed 2 × with Leibovitz's L-15 medium containing the experimental treatments and were left in the last wash for microscopy. The dye was excited with the 488 nm line of the confocal lasers. Laser intensity was kept at ~10% of maximum to reduce photo-oxidation of the dye. Several passes of the laser at this power did not significantly increase CM–$H_2$ DCFDA intensity indicating that little photo-oxidation occurred in the cells at this power. Laser intensity and photomultiplier gain were always the same within an experiment. The green photomultiplier channel of the confocal microscopes was used for image acquisition.

We used $TMRM^+$ in non-quench mode to monitor $\Delta\psi_m$ [25–27]. Cultures were incubated for 20–25 min at 35 °C in the appropriate experimental medium containing $TMRM^+$ (10 nM). At the end of this time, cultures were washed 2 × with L15 media containing the appropriate experimental treatments and $TMRM^+$. They were left in the last wash for confocal microscopy. The dye was excited with the 543 nm line of the confocal lasers. The TRITC photomultiplier channel of the confocal microscope was used for image acquisition. That $TMRM^+$ was being used in non-quench mode was confirmed by the diminution of dye intensity when cells were acutely treated with the protonophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 200 nM).

Both CM–$H_2$ DCFDA and $TMRM^+$ fluorescence intensities in confocal micrographs were quantified by measuring the raw pixel intensity in a 60 $\mu m^2$ area within individual neuronal somas using the elliptical region tool of MetaMorph software (Universal Imaging, West Chester, PA). All microscopy was done at room temperature. The intensity of each neuron was normalized to that of NGF-maintained $bax^{+/+}$ neurons receiving the same concentration of dye for the same time as the experimental cells.