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Bax Affects Production of Reactive Oxygen by the Mitochondria of Non-apoptotic Neurons

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Abstract

Depriving sympathetic neurons in cell culture of nerve growth factor (NGF) causes their apoptotic death. Bax-induced release of cytochrome *c* from mitochondria and the subsequent activation of cytosolic caspases are central to this death. A Bax-dependent increase of mitochondrial-derived reactive oxygen species (ROS) that is an important component of the apoptotic cascade in these cells begins soon after NGF withdrawal. Here we report that Bax can also influence mitochondrial production of ROS in non-apoptotic sympathetic neurons. We determined ROS levels by using confocal microscopy to monitor changes in the fluorescence intensity of a redox-sensitive dye loaded into single cells. ROS levels were similar in NGF-replete *bax* wild-type neurons and neurons from which *bax* had been deleted. To enhance any effects that Bax might have on ROS levels in NGF-replete cells we exposed cultures to the ATP synthase inhibitor, oligomycin. This treatment hyperpolarizes mitochondrial membrane potential ($\Delta\Psi_m$), an event that can favor increased ROS production. NGF-replete neurons from mice in which *bax* had been deleted had much higher levels of mitochondrial-derived ROS when treated with oligomycin than did *bax* wild-type cells. Oligomycin treatment also caused greater hyperpolarization of $\Delta\Psi_m$ in bax-deleted cells than in wild-type cells. These findings indicate that Bax can affect mitochondrial ROS production in non-apoptotic neurons and may do so by altering $\Delta\Psi_m$.

Sympathetic neurons undergo apoptotic death *in vivo* and *in vitro* when deprived of their required neurotrophic factor, NGF (Martin *et al*., 1988; Deckwerth and Johnson, 1993; Deshmukh *et al*., 1996; Neame *et al*., 1998; Martinou *et al*., 1999; Putcha *et al*., 2002). Apoptosis in these and many other types of neurons depends on the proapoptotic Bcl-2 family member, Bax (Deckwerth *et al*., 1996; Miller *et al*., 1997; White *et al*., 1998). Withdrawing NGF from sympathetic neurons causes Bax to become tightly associated with the outer membrane of their mitochondria where it induces release of cytochrome *c* and other apoptogenic substances from the mitochondrial intermembrane space into the cytoplasm (Liu *et al*., 1996; Wolter *et al*., 1997; Putcha *et al*., 1999; Susin *et al*., 1999; Du *et al*., 2000; Li *et al*., 2001). ROS levels are elevated in NGF-deprived sympathetic neurons in cell culture before they become committed to apoptotic death (Greenlund *et al*., 1995; Dugan *et al*., 1997). These ROS derive from the mitochondrial electron transport chain, lie downstream of Bax, and appear to be an important component of the apoptotic cascade (Kirkland *et al*., 2002). In the process of carrying out experiments on the effects of Bax on ROS in apoptotic neurons we discovered that Bax can also have a potent effect on production of ROS in non-apoptotic cells maintained in NGF-containing medium.

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Sympathetic neurons were obtained from the superior cervical ganglia of newborn mice. *bax* wild-type ($bax^{+/+}$), hemizygous ($bax^{+/-}$knockout ($bax^{-/-}$) mice were generated by mating mice hemizygous for the *bax* allele (Jackson Labs, Bar Harbor, ME; Knudson *et al*., 1995). Separate cultures were established for the ganglia from each pup of $bax^{+/-}$ X $bax^{+/-}$ matings. Genomic DNA was prepared from the tail of these pups and genotypes determined by PCR as described previously (Kirkland *et al.*, 2002). Dissociation of neurons from the ganglia and plating of neurons in cell culture dishes were also done as described (Franklin *et al*., 1995; Deckwerth *et al*., 1996; Kirkland *et al*., 2002). Neurons were plated on #1 glass coverslips. These were placed in an Attofluor cell chamber (Molecular Probes, Eugene, OR) for microscopy. Cultures were incubated at the standard temperature for culturing sympathetic neurons (35° C; Johnson and Argiro, 1983) in an incubator having a 5% CO_2 and 95% air atmosphere. They were maintained in Eagle's minimum essential medium with Earle's salts w/o L-glutamine (Life Technologies, Inc., Rockville, MD) 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 6–9 days old when used for experiments.

Relative ROS levels were determined by confocal microscopic imaging of neurons loaded with the redox-sensitive dye 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate (CM-H_2 DCFDA; Molecular Probes) as described (Royall and Ischiropoulos, 1993; Kirkland *et al*., 2002). The intensity of this dye increases primarily upon oxidation by H_2O_2 and free radical products downstream of H_2O_2. Nerve growth factor 2.5S was from Harlan Bioproducts (Indianapolis, IN). All other reagents were from Sigma (St. Louis, MO). Confocal microscopy was done with a Nikon C1 laser-scanning confocal system (Southern Micro Instruments, Marietta, GA) attached to a Nikon Eclipse TE300 inverted microscope. Image capture and data analysis was accomplished by EZC1 software. Neurons were scanned at 512 X 512 pixel resolution. The dye was excited with the 488 nm line of the confocal lasers and the FITC photomultiplier of the confocal microscope was used for image acquisition. Confocal pinhole size was always the same within an experiment.

While most Bax resides in the cytoplasm of non-apoptotic sympathetic neurons and other non-apoptotic cell types, a substantial fraction of the cellular Bax pool is associated with the outer mitochondrial membrane (Putcha *et al*., 1999, 2002; Kaufmann *et al*., 2004). To determine whether this pool of Bax affects basal ROS levels in NGF-replete cells, we measured CM-H_2DCFDA intensity in $bax^{+/+}$, $bax^{+/-}$, and $bax^{-/-}$ neurons maintained in NGF-containing media (Fig. 1). For each experiment the average CM-H_2DCFDA intensity of NGF-maintained, $bax^{+/+}$ neurons plated and loaded with dye at the same time as neurons of the other genotypes was used as control. $bax^{-/-}$ neurons do not have any detectable Bax protein while $bax^{+/-}$ cells have about half of the Bax concentration of $bax^{+/+}$ neurons (Kirkland *et al*., 2002). The average CM-H_2DCFDA intensity in the $bax^{-/-}$ neurons was 0.2 ± 0.07-fold less than the average in the $bax^{+/+}$ cells ($p = 0.013$). To attempt to augment any differences in ROS levels in NGF-replete neurons with each of the three *bax* genotypes, we treated cultures with the macrolide antibiotic, oligomycin (5 μg/ml). This compound blocks passage of protons through the mitochondrial $F_1.F_O$ ATP synthase. In normally functioning mitochondria, block of the synthase by oligomycin hyperpolarizes $\Delta\Psi_m$ by increasing the proton gradient across the inner membrane. ATP synthesis is also blocked. Hyperpolarization of $\Delta\Psi_m$ can increase leakage of electrons from the mitochondrial electron transport chain (Nicholls and Budd, 2000; Nicholls and Ward, 2002; Nicholls and Ferguson, 2002). These electrons then reduce molecular oxygen to superoxide which in turn is converted into ROS that can be detected by CM-H_2DCFDA. Oligomycin, used at a concentration known to block most ATP production in these cells (Chang *et al*., 2003) greatly increased CM-H_2DCFDA intensity in NGF-maintained $bax^{+/+}$ neurons (Fig. 1A, B). The ROS levels in $bax^{+/+}$ and $bax^{+/-}$ cells were increased by an equivalent amount ($p = 0.44$) by this treatment (Fig. 1A, B). Surprisingly, the oligomycin treatment caused

a much larger increase of ROS in the *bax*$^{-/-}$ neurons than in the other two genotypes. Average CM-H_2DCFDA intensity was about 2-fold higher in these cells than in the oligomycin-treated *bax*$^{+/+}$ and *bax*$^{+/-}$ neurons (about 5-fold higher than control; Fig 1A, B). Therefore, Bax concentration had a profound effect on ROS levels in NGF-replete cells treated with oligomycin. These differences cannot be explained by differential dye loading as the same amount of CM-H_2DCFDA loads into cells of each of the three *bax* genotypes (Kirkland *et al*., 2002).

To explore a possible relationship between $\Delta\Psi_m$ and ROS in the oligomycin-treated neurons, we used the $\Delta\Psi_m$-sensitive dye, tetramethylrhodamine methyl ester (TMRM+; Molecular Probes) in non-quench mode (Nicholls and Budd, 2000; Nicholls and Ward, 2002; Nicholls and Ferguson, 2002). Cultures were incubated for 20–25 min at 35° C in the appropriate experimental medium containing $TMRM^+$ (10 nM). Cultures were then washed 2X with Leibovitz's 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 of the confocal microscope was used for image acquisition. Fig. 1C, D shows that oligomycin increased $\Delta\Psi_m$ in neurons of each *bax* genotype. There were no differences in the $\Delta\Psi_m$ in cells with each *bax* genotype without oligomycin treatment. However, Bax concentration was inversely related to $\Delta\Psi_m$ in NGF-replete, oligomycin-treated neurons. The most hyperpolarized (highest) $\Delta\Psi_m$ was found in *bax*$^{-/-}$ cells while the lowest $\Delta\Psi_m$ was in *bax*$^{+/-}$ and *bax*$^{+/+}$ cells. Therefore, higher Bax concentrations were associated both with lower ROS levels and lower $\Delta\Psi_m$ in oligomycin-treated cells. These findings suggest that Bax had a depolarizing effect on $\Delta\Psi_m$ in NGF-replete sympathetic neurons and that the augmentation of mitochondrial ROS production by *bax* deletion in oligomycin-treated cells may have been secondary to mitochondrial hyperpolarization resulting from the absence of this depolarizing effect.

The effect of Bax concentration on ROS in the NGF-replete neurons was the opposite of that observed in NGF-deprived cells where higher Bax protein concentration is associated with elevated ROS levels (Kirkland *et al*., 2002). One possible explanation for this apparent discrepancy is that, during apoptosis, Bax-induced release of cytochrome *c* from the mitochondria causes activation of caspases that then attack mitochondrial respiratory complexes causing increased ROS production (Ricci *et al*., 2004). However, Bax also has pro-oxidant effects in apoptotic neurons that are separable from effects related to cytochrome *c* release (Kirkland and Franklin, 2001; Kirkland *et al*., 2002). Another possible explanation for the apparent discrepancy is that Bax also has a pro-oxidant effect in NGF-replete neurons causing them to upregulate antioxidant defense mechanisms. This possibility is suggested by the slightly elevated ROS in *bax*$^{+/+}$ cells as compared to *bax*$^{-/-}$ neurons. While the suppression of basal ROS by *bax* deletion did not achieve high statistical significance, it is possible that it reveals a trend that would be made more apparent with additional data. The elevated ROS observed in oligomycin-treated *bax*$^{-/-}$ as compared to *bax*$^{+/+}$ neurons might then be explained by a diminished ability of the former neurons to detoxify increased ROS. However, neurons of all three genotypes exhibit an identical, linear relationship between H_2O_2 concentrations and CM-H_2DCFDA intensities over the range of CM-H_2DCFDA intensities reported here (Kirkland *et al*., 2002). This finding suggests it is unlikely that the observed differences in ROS levels in neurons of the three *bax* genotypes can be explained by differences in their abilities to detoxify ROS. Moreover, the data presented here shows that oligomycin increased $\Delta\Psi_m$ more in the *bax*$^{-/-}$ than in the *bax*$^{+/+}$ neurons which cannot be explained by differences in cellular antioxidant capacity. Elevated production of ROS by mitochondria is favored by increased $\Delta\Psi_m$ because of thermodynamic considerations (Nicholls and Budd, 2000; Nicholls and Ward, 2002; Nicholls and Ferguson, 2002). Therefore, the most likely explanation for the effects of Bax deletion on elevated ROS in the oligomycin-treated neurons is that Bax influences $\Delta\Psi_m$. How Bax could exert this type of effect is unknown. Bax causes mitochondrial

depolarization in digitonin-permeabilized astrocytes (Carvalho *et al*., 2004). This effect may be secondary to Bax-induced depletion of cytochrome *c* from the mitochondrial electron transport chain. That cannot be the explanation for the data reported here as the cells were not apoptotic and Bax levels should be unrelated to amount of cytochrome *c* retained in mitochondria. Confirming this statement, we have found that there are no differences in the amounts of cytochrome *c* in neurons with each of the three *bax* genotypes (data to be reported elsewhere). Therefore, Bax can affect $\Delta\Psi_m$ and ROS levels even in non-apoptotic sympathetic neurons. How Bax does this is unknown but could involve effects on mitochondrial respiration or on the proton gradient of the inner mitochondrial membrane.

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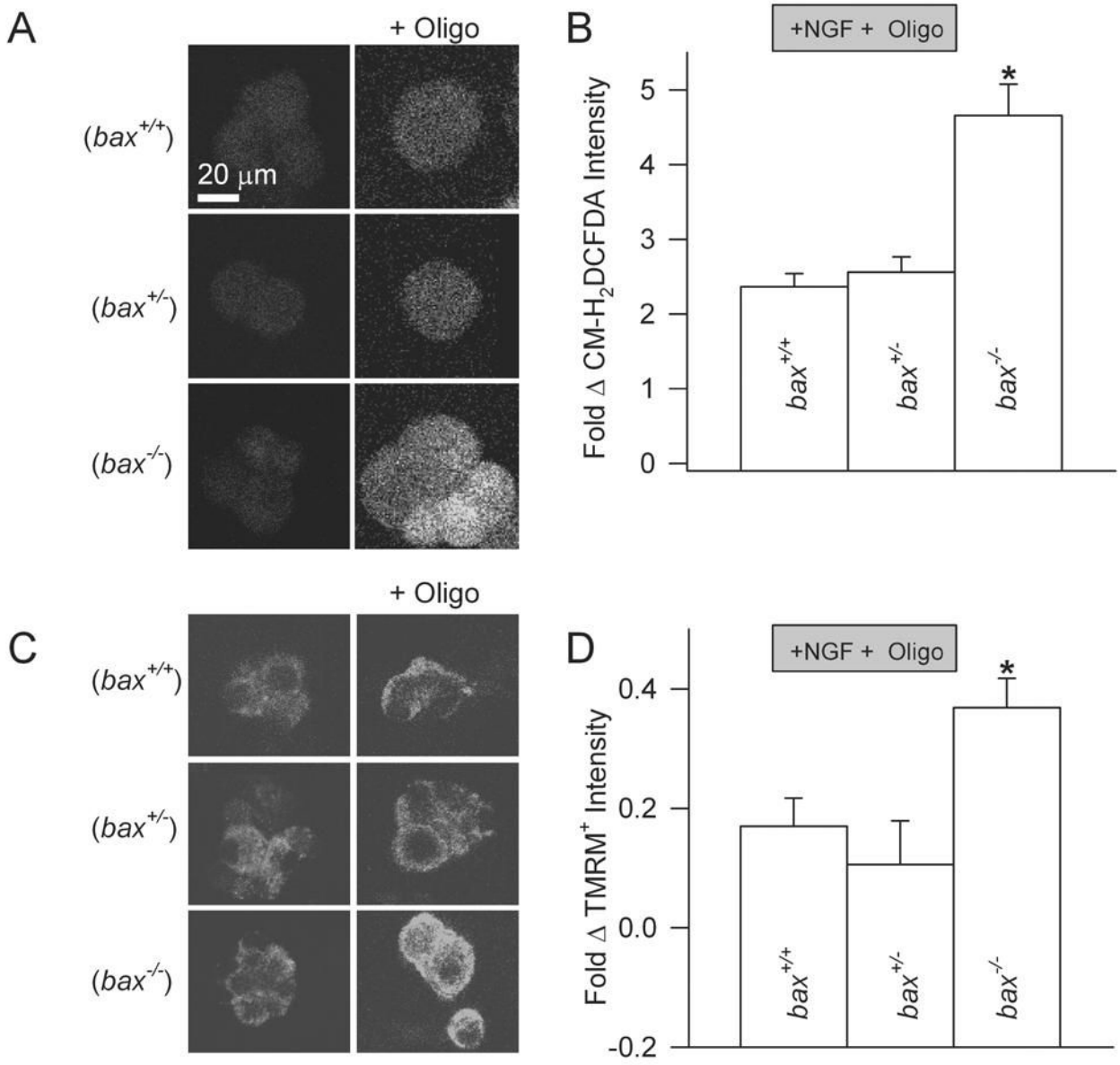

Fig. 1. Oligomycin increased both ROS and $\Delta\Psi_m$ more in NGF-replete *bax*$^{-/-}$ neurons than in neurons with *bax*$^{+/+}$ and *bax*$^{+/-}$ genotypes

(A) Confocal micrographs showing NGF-replete neurons loaded with the redox-sensitive dye, CM-H_2DCFDA. Left images show the very faint staining observed in *bax*$^{+/+}$, *bax*$^{+/-}$, and *bax*$^{-/-}$ neurons maintained in NGF-containing medium. Right images show neurons of the same three genotypes loaded with CM-H_2DCFDA and exposed to Oligomycin (5 μg/ml). Note the increase in dye fluorescence compared to the neurons on the left indicating that the dye is oxidized.

(B) In NGF-replete cells, oligomycin (5 μg/ml) elevated ROS (CM-H_2DCFDA intensity) to equivalent levels in *bax*$^{+/+}$ and *bax*$^{+/-}$ neurons (p = 0.44). However, it increased ROS much more in NGF-replete *bax*$^{-/-}$ neurons than in neurons of the other two genotypes (p = 0.004 compared to the increase in *bax*$^{+/+}$ cells). Treatment with oligomycin was done during the 20 min of incubation with CM-H_2DCFDA. Oligomycin was also included in the L15 medium in

which the cells were maintained during microscopy. n = 150–183 neurons. All experiments were done at least three times with neurons from at least three separate platings.

(C) Confocal micrographs of neurons loaded with the $\Delta\Psi_m$–dependent dye, $TMRM^+$ (10 nM continuous exposure). Left images show NGF-replete $bax^{+/+}$, $bax^{+/-}$, and $bax^{-/-}$ neuronal somas loaded with $TMRM^+$. Right images show NGF-replete $bax^{+/+}$, $bax^{+/-}$, and $bax^{-/-}$ neurons loaded with $TMRM^+$ while also exposed to oligomycin (5 μg/ml). The increase in $TMRM^+$ fluorescence intensity indicates hyperpolarization of $\Delta\Psi_m$.

(D) Oligomycin (5 μg/ml) increased $\Delta\Psi_m$ to similar levels in NGF-replete neurons having either $bax^{+/+}$ or $bax^{+/-}$ genotypes ($p = 0.47$) but had a greater effect on $bax^{-/-}$ cells ($p < 0.0001$ compared to the increase in $bax^{+/+}$ cells). Neurons were incubated for 20 minutes with oligomycin and $TMRM^+$ (10 nM). Both compounds were also included in the L15 medium used for microscopy. n = 133–180 neurons. All experiments were done at least three times with neurons from at least three separate platings.