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Prooxidant Effects of NGF Withdrawal and MEK Inhibition in Sympathetic Neurons

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ABSTRACT

An increase of mitochondrial-derived reactive oxygen species (ROS) occurs in nerve growth factor (NGF)-deprived sympathetic neurons undergoing apoptotic death. It has been reported that NGF suppresses increased ROS production by the mitochondria in these cells through a mitogen-activated protein kinase kinase (MEK)/mitogen-activated protein (MAP) kinase pathway because NGF withdrawal inactivates this pathway and the MEK inhibitor, PD98059, increases ROS in the presence of NGF. We show here that treating rat sympathetic neurons in cell culture with PD98059 greatly decreased cellular concentrations of reduced glutathione (GSH), a major cellular antioxidant. Therefore, it is likely that this inhibitor induces a cellular prooxidant state in NGF-maintained sympathetic neurons primarily by decreasing GSH concentration rather than by causing increased mitochondrial ROS production. These data suggest that the MEK/MAP kinase signaling pathway regulates cellular GSH concentration. *Antioxid. Redox Signal.* 5, 635–639.

INTRODUCTION

Approximately 50% of neurons produced during the embryogenesis of the vertebrate nervous system die by apoptosis before birth or shortly thereafter (10). Cells obtaining a sufficient quantity of a required neurotrophic factor survive the period of developmental death. The classic model system for investigating the molecular mechanisms underlying this death consists of nerve growth factor (NGF)-deprived rat or mouse sympathetic neurons in cell culture (1, 5, 9, 14). None of these neurons become committed to apoptotic death until ~18 h after NGF withdrawal. During the period before commitment, cells enter a prooxidant state caused by increased levels of reactive oxygen species (ROS) derived from the mitochondrial electron transport chain (2, 6–8). Evidence suggests that these ROS are a critical component of the apoptotic death of these cells. Dugan *et al.* (2) reported that adding NGF back to the culture medium after withdrawal causes a rapid block of increased ROS. Additionally, they found that the mitogen-activated protein kinase kinase (MEK) inhibitor, PD98059, blocked the ability of NGF to suppress elevated ROS and suggested that the NGF suppression of ROS was mediated via a MEK/mitogen-activated protein (MAP) kinase pathway. We show here that PD98059 caused a profound decrease of reduced glutathione (GSH) in rat sympathetic neurons under all culture conditions and that this decrease likely accounts for the prooxidant effects of PD98059. Therefore, we conclude that the increased ROS caused by PD98059 treatment does not indicate that NGF suppresses increased mitochondrial-derived ROS during neuronal apoptosis via a MEK/MAP kinase pathway.

MATERIALS AND METHODS

Materials

Monochlorobimane (MCB) and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluoresceindiacetate (CM-H_2DCFDA) were from Molecular Probes (Eugene, OR, U.S.A.). NGF 2.5S was from Harlan Bioproducts (Indianapolis, IN, U.S.A.). All other reagents were from Sigma (St. Louis, MO, U.S.A.).

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Culture of rat sympathetic neurons

Superior cervical ganglia were dissected from Sprague–Dawley rat fetuses (Harlan Bioproducts) on embryonic day 20 or 21. Neurons were enzymatically and mechanically dissociated from the ganglia and maintained in cell culture as described (5, 7). Culture medium consisted of Eagle's minimum essential medium with Earle's salts (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μ*M* fluorodeoxyuridine,20 μ*M* uridine, 1.4 m*M* L-glutamine, and 50 ng/ml 2.5S NGF. Cells used in survival assays were plated on a collagen substrate in 24-well Costar tissue culture dishes (Corning, Inc., Corning, NY, U.S.A.). Those used for fluorescent or confocal microscopy were plated on a collagen substrate coated on glass coverslips glued over holes cut in the bottoms of 35-mm Falcon tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). One-half to one ganglion was plated per culture in all experiments. NGF was withdrawn from cells by incubating cultures in the culture medium containing a NGF-neutralizing antibody (Harlan Bioproducts) and lacking NGF. Cells had been in culture for 6–9 days at the beginning of experiments.

Confocal and fluorescence microscopy

Confocal imaging was done with a Bio-Rad MRC 1024 laser scanning confocal microscope mounted on a Nikon Diaphot 200 inverted microscope as described (7). The confocal microscope was controlled by 24-bit MRC-1024 Laser Sharp Software (version 3.0; Bio-Rad, Hercules, CA, U.S.A.) running on a Compac Prosignia 300 computer. Neurons were visualized with a 60×-plan oil immersion lens (N.A. 1.4). Cells were scanned by the confocal microscope at 512 × 512 pixel resolution.

Fluorescence microscopy was done with a Nikon TE300 inverted microscope equipped with a xenon lamp as described (7, 8). Images were captured by a CCD camera (MicroMAX; Princeton Institute, Trenton, NJ, U.S.A.) using Metamorph software (Universal Imaging Co., West Chester, PA, U.S.A.). All microscopy was done at room temperature.

ROS measurement

We determined cellular ROS levels by measuring relative intensities of the redox-sensitive dye CM-H_2DCFDA. This dye is membrane-permeant, is trapped in cells by binding onto the chloromethyl groups of cellular thiols, and fluoresces when oxidized by hydrogen peroxide and its downstream free-radical products (12). We extensively characterized the properties of this dye in rat and mouse sympathetic neurons and in mouse cerebellar granule cells (7, 8). These characterizationsshow that CM-H_2DCFDA is well retained by these cells and is resistant to photooxidation. Control experiments demonstrated that none of the data presented here can be explained by differential dye loading, pH effects on the dye, or by alterations in the ability of the dye to respond to oxidation with increased fluorescence.

Cultures were incubated with CM-H_2DCFDA (10 μ*M*) for 20–25 min at 35°C in medium containing the experimental treatments. Cultures were then washed two or three times with Leibovitz's L-15 medium containing the experimental reagents. They were left in the last wash for confocal microscopy. The 488-nm line of the confocal laser excited the dye, and the FITC photomultiplier was used for image acquisition. Quantification of CM-H_2DCFDA intensity was accomplished with Sigmagel software (SPSS Science, Chicago, IL, U.S.A.) by measuring raw pixel intensity in a 60-μm^2 area of the neuronal soma. The CM-H_2DCFDA intensity of each neuron was normalized as described (7) to that of the average CM-H_2DCFDA intensity of control neurons maintained in medium containing NGF. Publication images were prepared with Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, CA, U.S.A.).

GSH assay

Cellular levels of GSH were estimated by fluorescence microscopic imaging of single cells stained with MCB, a dye that fluoresces when bound to GSH (3, 7, 8). We previously determined that MCB staining intensity accurately reflects GSH concentration measured in cultures of rat sympathetic neurons by an enzymatic technique (7). Cultures were exposed to MCB (5 μ*M*) in L-15 medium for 30 min at room temperature. Cultures were then washed twice with L-15 medium lacking MCB. MCB was excited at 380 ± 15 nm. Emission filter was 510 ± 20 nm. MCB intensity was quantified with Metamorph software. Intensities were normalized to average MCB intensities measured in NGF-maintained cells plated at the same time as the experimental cells.

Statistics

Statistical analysis was done with SigmaStat 2.0 (SPSS). Statistical comparisons were made with Kruskal–Wallis one-way ANOVA on ranks with Dunn's multiple comparisons. Means in all figures are shown as ± SEM.

RESULTS

As previously reported (2), application of PD98059 to NGF-maintained rat sympathetic neurons in cell cultures caused rapid elevation of cellular ROS levels (data not shown). This increase was maintained for at least 24 h (Fig. 1). The antioxidant *N*-acetyl-L-cysteine (LNAC) blocks increased ROS in these cells after NGF withdrawal and inhibits apoptosis (7). Decreased ROS and apoptosis in NGF-deprived, LNAC-treated cells is caused, in part, by an LNAC-mediated increase in cellular GSH concentration. Treatment of NGF-deprived, LNAC-maintained neurons with PD98059 greatly attenuated the ability of LNAC to block increased ROS (Fig. 1; $p < 0.001$). The protein synthesis inhibitor cycloheximide (CHX; 1 μg/ml) blocks all apoptosis in these cells for several days after NGF deprivation. It also increases cellular GSH concentration, probably by shunting cysteine from incorporation into protein into GSH synthesis (11). Treatment of NGF-deprived cells with 1 μg/ml CHX completely prevents increased ROS after NGF withdrawal. The block of ROS is secondary to the increased GSH (7). Treatment of NGF-deprived cultures with PD98059 attenuated the ability of CHX to block the ROS burst (Fig. 1B). Therefore, under all experimental conditions tested, PD98059 increased cellular ROS levels ($p < 0.001$ compared with NGF control).

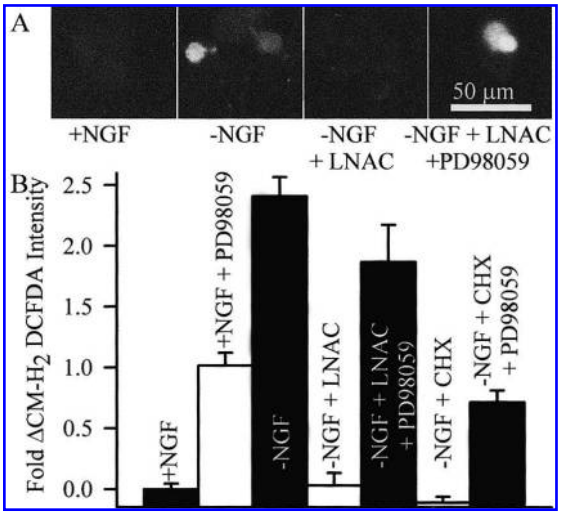

FIG. 1. The MEK kinase inhibitor, PD98059, increased cellular ROS levels in rat sympathetic neurons in cell culture. (**A**) Fluorescent micrographs of rat superior cervical ganglia neuronal somas loaded with the redox-sensitive dye, CM-H_2DCFDA. Increased CM-H_2DCFDA intensity after 24 h of NGF deprivation indicates elevated ROS levels. The antioxidant, LNAC (30 m*M*), blocked increased ROS caused by NGF withdrawal. The ability of LNAC to prevent elevated ROS was blocked by PD98059 (100 μ*M*; a concentration that blocks all MEK/MAP kinase activity in these cells). (**B**) Quantification of the effects of PD98059 (100 μ*M*) on cellular ROS levels. LNAC (30 m*M*) and CHX (1 μg/ml) blocked elevated ROS after NGF deprivation. PD98059 prevented LNAC from blocking the ROS increase after NGF withdrawal and greatly attenuated the ability of CHX to do so. PD98059 also increased ROS levels in NGF-maintained cells. Neurons were maintained under the indicated conditions for 24 h. $n = 85–113$ neurons from three separate platings.

PD98059 only slightly decreased the ability of NGF to support survival (Fig. 2). However, it completely blocked the ability of LNAC to promote survival in the absence of NGF and greatly attenuated the ability of CHX to block apoptosis. Because both LNAC and CHX block increased ROS in NGF-deprived cells by increasing cellular GSH concentration (7), the most likely explanation for the effect of PD98059 on ROS and survival is that it blocked increased GSH. To test this hypothesis, we determined relative levels of GSH. Figure 3 shows that, as previously reported (7), withdrawal of NGF causes increased cellular GSH ($p < 0.001$ compared with NGF-replete control). The mechanism underlying this GSH increase is unknown. Adding LNAC or CHX to the culture medium further increased GSH levels. PD98059 blocked the increased GSH caused by LNAC and CHX ($p < 0.001$). It also reduced GSH levels in control cells maintained in NGF. Thus, it seems likely that PD98059 increased ROS by decreasing cellular GSH levels and that it inhibited the ability of LNAC and CHX to prevent apoptosis because it blocked their antioxidant properties. PD98059 increased ROS and reduced GSH in NGF-maintained cells, but only caused a small decrease in survival. This was likely due to prosurvival effects of NGF that are unrelated to cellular redox state.

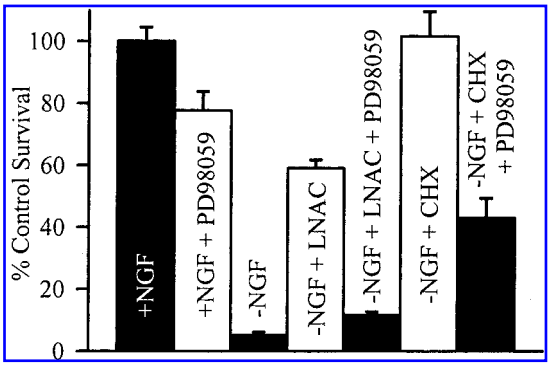

FIG. 2. Effects of PD98059 on neuronal survival. PD98059 (100 μ*M*) slightly, but significantly ($p < 0.01$ compared with +NGF control) decreased the ability of NGF to support survival. The ability of LNAC (30 m*M*) to promote survival was completely blocked by the same concentration of PD98059 ($p > 0.01$ compared with −NGF control), and the ability of CHX (1 μ*M*) to do so was greatly attenuated ($p < 0.01$ compared with −NGF + CHX control). The effect of PD98059 on survival was determined in cells deprived of NGF and exposed to the indicated agents for 3 days. The medium was then replaced with NGF-containing medium and survival determined by blinded cell counting at least 5 days later. $n = 14–17$ cultures from three separate platings.

DISCUSSION

Similar to the findings reported here in sympathetic neurons, Yan and Greene (13) found that PD98059 blocks the ability of LNAC to inhibit apoptosis in PC-12 cells. They also showed that LNAC can activate MAP kinase in these cells. Because of these findings, they suggested that LNAC promotes survival of PC-12 cells through an effect on this pathway rather than on cellular redox state. However, our results suggest that MEK/MAP kinase activity, by itself, can affect cellular redox state by altering GSH concentration. Therefore, it is likely that LNAC does affect survival through an antioxidant mechanism.

Inhibition of MEK by PD98059 attenuated the ability of NGF to support survival of sympathetic neurons and greatly decreased the ability of CHX to prevent apoptosis in NGF-deprived cells. In each case, this compound also decreased cellular GSH concentration and the ability of these agents to suppress ROS. Addition of NGF to NGF-deprived sympathetic cultures quickly suppresses apoptosis by causing a rapid inhibition of cytochrome *c* release from mitochondria (7, 8). It also rapidly suppresses increased ROS (2). However, neither effect appears to involve regulation of GSH by NGF. It is surprising that GSH levels actually increase in NGF-deprived rat sympathetic neurons (7) even though MEK/MAP kinase activity is greatly diminished. We have no ready explanation for why blocking MEK/MAP kinase activity with PD98059 decreases cellular GSH concentration, whereas diminishing MEK/MAP kinase activity by NGF withdrawal is associated with increasing GSH. It is possible that other pathways are activated on NGF withdrawal that compensate for the decreased MEK/MAP kinase activity and cause the increased GSH levels. Regardless of the explanation, taken together the data suggest that suppression of the MEK/MAP kinase pathway can, in some circumstances,

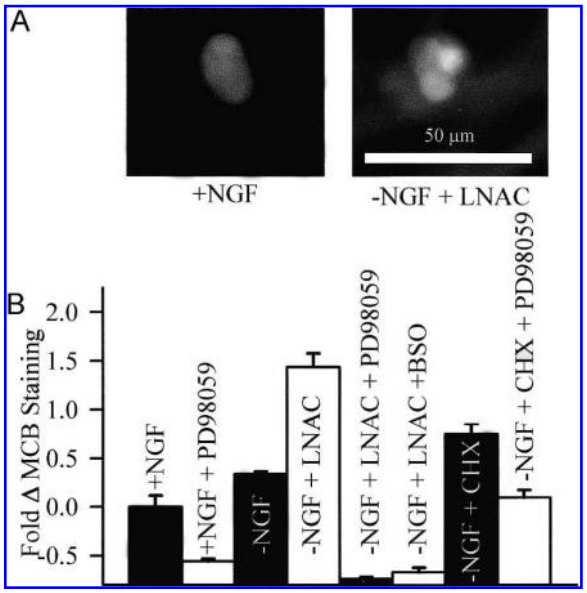

FIG. 3. PD98059 decreased cellular GSH levels. (**A**) Fluorescent micrographs showing the neuronal somas of rat superior cervical ganglia stained with the GSH-sensitive dye, MCB. Increased MCB intensity in NGF-deprived cells exposed for 24 h to LNAC (30 m*M*) are brighter than control cells, suggesting higher GSH concentrations. (**B**) Quantification of the effects of PD98059 (100 μ*M*) on cellular GSH levels. As previously reported, NGF deprivation caused increased GSH concentration (7). LNAC (30 m*M*) and CHX (1 μg/ml) further increased GSH after NGF withdrawal. PD98059 prevented GSH increase under all culture conditions tested. Buthionine sulfoximine (BSO; 200 μ*M*), an inhibitor of GSH synthesis, completely blocked increased MCB intensity caused by LNAC ($p < 0.001$), indicating that the MCB was detecting GSH. Neurons were maintained under the indicated conditions for 18–24 h. n = 37–154 neurons from two or three separate platings.

affect cellular redox state by regulating GSH levels. This regulation could occur either through MEK/MAP kinase effects on recycling of oxidized glutathione to GSH or on glutathione synthesis.

It is generally assumed that CHX blocks apoptosis because it prevents translation of proapoptotic proteins. The concentration of CHX used in the experiments reported here causes complete long-term block of protein synthesis in sympathetic neurons (4). Because PD98059 greatly attenuated the ability of CHX to block increased ROS, apoptosis (60% suppression of CHX-mediated saving), and GSH, the data suggest that suppression of protein synthesis by CHX or other agents is antiapoptotic, in part, because of antioxidant effects rather than block of production of killer proteins. However, CHX was a significantly more potent antiapoptotic agent than was LNAC (Fig. 2) even though LNAC increased GSH by a greater amount (Fig. 3). Thus, CHX had death-suppressant effects in addition to those resulting from increased GSH concentration. Therefore, our data do not preclude a role for *de novo* protein synthesis in the death of these cells.

ACKNOWLEDGMENTS

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ABBREVIATIONS

CHX, cycloheximide; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluoresceindiacetate; GSH, reduced glutathione; LNAC, *N*-acetyl-L-cysteine; MAP, mitogen-activated protein; MCB, monochlorobimane; MEK, mitogen-activated protein kinase kinase; NGF, nerve growth factor; ROS, reactive oxygen species.

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