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INTRODUCTION

Apoptosis has been shown to contribute to neuronal loss in Parkinson's Disease (PD) and after a number of forms of toxic exposure. If unique elements in the signaling pathways for the PD or toxic apoptosis can be identified and their apoptosis signaling impeded, neuronal loss may be slowed or reduced in the conditions. The research proposed in this grant was designed to examine the role of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in apoptotic neuronal signaling. Recent studies in postmortem brain have implicated GAPDH apoptosis signaling in Parkinson's disease (PD). Propargylamines, with structural similarity to (-)-deprenyl, bind to GAPDH and convert the protein from a tetrameric to a dimeric form in association with a decrease in apoptosis.

We have examined GAPDH-related mechanisms underlying propargylamine anti-apoptosis and have found that the propargylamines induce changes in the synthesis of a number of proteins, including changes that are appropriate to prevent increases in mitochondrial membrane permeability (MMP). Increases in MMP lead to the release of a number of mitochondrial factors that signal for the final stages of apoptotic degradation. We also found that the alterations in new protein synthesis induced by propargylamines are identical to those resulting from the exposure of cells to non-toxic levels of pro-oxidants. The propargylamine induced anti-apoptosis is dependent on pathways that involve phosphatidylinositol 3-kinase (PI3-K). It remains to be determined how conversion GAPDH from a tetramer to a dimer induces transcriptional activity that protects neurons from apoptosis. Our studies have also shown that progesterone and estradiol are anti-apoptotic and share PI3-K dependency with the propargylamines and likely an association with GAPDH.

We reported that GAPDH signaling contributed to some forms of cellular apoptosis but not others. MPTP, acting through its metabolite MPP+, induces PD-like, toxic loss of catecholaminergic (CAergic) neurons in animals and humans. We and others have provided evidence that MPP+ induced apoptosis involves GAPDH signaling. MPTP mimics PD neuronal loss primarily due to MPP+ uptake into CAergic neurons by the dopamine membrane transporter (DAT). MPP+ does not cause the protein aggregations in CAergic neurons similar to the Lewy bodies, which are pathognomonic of PD.

Decreased mitochondrial respiratory complex I (mtRCx I) activity has been suggested to underlie PD. MPP+ binds to mtRCx I and decreases mtRCx I activity, suggesting a possible mechanistic link between MPP+ and PD. Recent studies have shown that MPP+ also binds to the vesicular monoamine transporter (VMAT2). The VMAT2 binding, rather than mtRCx I binding, may be responsible for MPP+ induced CAergic loss so that MPTP may not mimic the decreased mtRCxI activity found in PD. Recently rotenone, a plant derived mtRCx I inhibitor has been reported to induce PD-like neuronal loss and Lewy body-like neuronal protein aggregations in rats. Rotenone may offer a mtRCx I dependent model of PD. We have found that rotenone induced decreases in mtRCx I activity caused changes in a number of apoptosis signaling proteins, particularly GAPDH, that appear similar to those in PD. Furthermore, rotenone induced apoptosis in CAergic neurons is markedly reduced by treatment with deprenyl-related propargylamines that bind to GAPDH.

BODY OF WORK

Background To Statement of Work

Similarities And Differences Between PD And MPTP Parkinsonism. Sporadic PD is characterized by CAergic neuronal loss, including melanin-containing, dopaminergic neurons (DAns) in the substantia nigra (SNc) and noradrenergic neurons in the locus coeruleus (LC). The pathobiology of the initiation and progression of PD remains uncertain (see [1] for a review). Stereological counting has shown that SNc melanin-containing DAns are decreased by 60% or more before PD becomes clinically evident PD [2, 3].

MPTP kills SNc DAns in non-human primates [4-6] as well as other CAergic neurons [7-10] with a similar, but not identical, regional distribution to human PD [11]. MPTP does not induce neuronal cell body protein aggregations similar to the Lewy bodies found in PD. Lewy bodies are a key-identifying feature of PD and contain a variety of proteins including ubiquitin, α-synuclein and GAPDH [12-14].

Decreased mtRCxI Activity In PD And MPTP Parkinsonism. Examination of homogenates from PD postmortem SNc revealed decreases of 20 to 40% in mtRCx I activity without similar activity decreases for complex II, III or IV [15-19]. Western blots and immunocytochemistry using a mtRCx I antibody revealed decreased immunoreaction for PD SNc [15, 18, 20, 21], possibly suggesting a deficiency of mtRCx I protein(s).

MPTP is converted to MPP^+ in astroglia by MAO-B [22, 23]. MPP^+ selectively accumulates in CAergic neurons by the plasma membrane dopamine transporter (DAT) [24, 25]. Inside CAergic neurons, MPP^+ undergoes high affinity uptake by the vesicular monoamine transporter (VMAT2) [26-28]. It binds less avidly to mtRCxI, where it inhibits NADH dehydrogenase [29-31]. The majority of MPP^+ appears to reside in CAergic vesicles rather than mitochondria [32]. Imaging studies have shown that ROS production caused by MPP^+ treatment of cultured DAns can results from vesicular release of dopamine rather than mtRCx I inhibition [33, 34]. Studies in nigral slices [35] and those in marmosets have similarly suggested that mtRCx I inhibition does not contribute to the pathology induced by MPTP [36]. In further support of the predominant role for vesicular dopamine release, VMAT2 overexpressing cells maintain dopamine in vesicles and resist MPP^+ [37, 38], while VMAT2 knockout mice are susceptible to MPP^+ [39, 40]. Accordingly, the selective involvement of CAergic neurons in MPTP parkinonism may result primarily from MPP^+ uptake by the DAT and not from MPP^+ inhibition of mtRCx I [34].

Neuronal Loss By Apoptosis In PD. Neuronal death can be broadly divided into two processes, necrosis and apoptosis. Morphologically, necrosis involves rapidly progressive cellular disruption marked by cell swelling, organelle disintegration, plasma membrane fracture, and cytoplasmic extrusion. In contrast, apoptosis proceeds more slowly and involves cellular degradation rather than disruption. Apoptotic degradation features cell shrinkage, plasma membrane blebbing, nuclear chromatin condensation, nuclear DNA fragmentation, maintenance of plasma membrane integrity but a loss of phospholipid asymmetry, cytoskeletal digestion, and the formation of membrane wrapped cytoplasmic and nuclear bodies. The degradation avoids the spilling of cytoplasmic contents into the extracellular space and primes cells for macrophage ingestion, thus preventing immune responses that injure neighbouring cells.

Concepts of apoptotic degradation have changed relatively little since the original descriptions thirty years ago [41, 42]. The degradation shows some variation in morphology from one form of apoptosis to another but is largely independent of cell type and the insult that initiates the process [43]. Although the degradation can be used to recognize apoptosis, it does not define the numerous signaling pathways, which set the stage for degradation and can differ markedly from one form of apoptosis from another. Over the last several years a myriad of molecules have been shown to operate within specific subcellular decisional networks that can be either pro- or anti-apoptotic (see [44] for a detailed and exciting review of organelle based signaling molecules in apoptosis). A variety of cysteine proteases (caspases) and molecules that induce or prevent increases in mitochondrial

membrane permeability function as the critical arbiters or integrators in apoptosis - they signal whether to degrade or not to degrade.

Is apoptosis involved in the neuronal loss found in PD? The use of *in situ* end labeling (ISEL), also termed as TdT dUTP nick end labeling (TUNEL), provided the initial means to recognize apoptotic nuclear DNA cleavage in neurons in PD postmortem brain but its use also engendered five years of controversy. Evidence for neuronal apoptosis in PD postmortem brain initially depended on nuclear DNA cleavage shown with ISEL [45], which appeared consistent with the subsequent finding of nuclear ultrastructural changes typical of apoptosis with electron microscopy [46]. But ISEL also seemed to rule against neuronal apoptosis in PD by the findings of : 1) similar numbers of ISEL positive nuclei in PD brain relative to controls [47]; 2) ISEL positive nuclei in glia rather than neurons [48]; and 3) large numbers of ISEL positive nuclei in control brains [49].

The conflicting evidence likely resulted from technical problems with the terminal transferase (TdT) used in ISEL (see [50] for details). TdT can label both single strand and double strand DNA breaks and can label nuclei in necrotic cells as well as apoptotic cells [51]. Depending on tissue preparation and ion concentrations, it can provide either false negatives or false positives and therefore cannot be used reliably to identify apoptotic cells.

Part of the interpretive difficulties were overcome by the application of dual labeling techniques that allowed for the simultaneous demonstration of DNA cleavage and chromatin condensation in the same nucleus. Nuclear DNA cleavage and chromatin condensation are mediated by different signaling events [52, 53]. Chromatin condensation can be readily visualized with fluorescent DNA binding dyes (see [54, 55] for our use of the dyes). The joint use of fluorescence ISEL and fluorescent DNA binding dyes allows for the demonstration of nuclear DNA fragmentation and chromatin condensation in the same nucleus [14, 50, 56]. If both are present in the same nucleus, it is virtually certain that the nucleus is undergoing apoptotic degradation. The joint labeling did not reveal high levels of apoptotic degradation in control brains or in glia in PD brains found with ISEL alone. It did show apoptotic degradation in a proportion of nigral neuromelanin containing neurons that seemed consistent with rates of loss of the neurons found in PD [14]. The number of neuromelanin-containing neurons in the PD SNc containing nuclei, which were both ISEL positive and chromatin condensation positive were about 300 times as frequent as those in the age-matched controls [14, 56].

Further conclusive evidence for apoptosis in PD was provided by two independent groups at about the same time. They demonstrated increased neuronal levels of activated caspase 3, the so called executioner caspase, in PD postmortem nigra [14, 57]. Caspase 3 is a key apoptosis signaling protein and is only found in apoptotic cells [43]. Other key pro-apoptotic signaling proteins have also been demonstrated in PD nigral neurons including Bax [58,14], caspase 8 [59], p53 [60], Fas and FasL [61, 62] and the dense nuclear accumulation of GAPDH [14]. Together these findings strongly support the view that apoptosis contributes to neuronal loss in PD and open the door to studies that fully define the apoptosis signaling pathways in PD. A complete understanding of the signaling pathways may provide insights into the cellular insults or defects that initiate the disease. Defining the signaling pathways may also offer new therapeutic approaches in PD since apoptosis-based therapies for a variety of diseases, including CGP3466 in PD (see below), are now in clinical trial (see [63] for a recent review).

Rotenone As A Potential Model Of Decreased mtRCx I Activity In PD. Rotenone, a pesticide, which occurs naturally in plants like thistles, specifically and potently reduces mtRCx I activity by inhibiting NADH dehydrogenase [64]. Animals that ingest plants containing rotenone can develop a nigropallidal, PD-like neurodegenerative condition [65] and epidemiological studies have implicated pesticides like rotenone in the increasing incidence of PD [66-68]. Rotenone is highly lipophilic so that it might be expected to cause a generalized decrease in mtRCx I activity that would not selectively damage or kill CAergic neurons and thereby mimic PD.

Injections of rotenone into the rat medial forebrain bundle, which carries the axons of SNc DAns projecting to the striatum, resulted in a depletion of striatal dopamine and dopamine metabolites, possibly as a result of selective dopaminergic axonal damage by rotenone [69]. Acute or subacute intravenous delivery of high rotenone dosages (14 - 24 mg/kg/day) to rats caused loss of neurons in the striatum and the globus pallidus, but not in the SNc [70]. The striatal damage involved focal regions of astrogliosis shown by increased glial fibrillary acidic protein (GFAP) immunoreaction with preservation

of GABA-ergic neurons, which form the majority of striatal neurons. Acute or subacute intravenous delivery of rotenone (5-15 mg/kg) to mice produced transient increases in striatal dopamine metabolites without altering dopamine levels [71], which suggested increased dopamine turnover without DAergic terminal loss. The subchronic intravenous administration of low rotenone doses (1.5 mg/kg, 3 times over 1 week) to the mice did not alter any dopamine indices.

Rotenone was also intravenously delivered to rats using Alzet osmotic minipumps at concentrations of 2 to 3 mg/kg/day over 7 to 35 days. Approximately 50% of surviving rats developed PD-like movement deficits together with pathological changes that included loss of SNc DAns and SNc neuronal protein aggregations with similarity to Lewy bodies [72]. The DAn loss was accompanied by focal areas of decreased striatal TH immunoreactivity, a marker of DAergic terminals. There was preservation of GAD and acetyl cholinesterase immunoreaction indicating that GABA-ergic and cholinergic neuronal populations were unaffected by rotenone. The prolonged low dose intravenous rotenone induced ubiquinated cell body protein accumulations in a proportion of surviving SNc DAns. The accumulations were α-synuclein immunopositive and shared features with PD Lewy bodies. Together with the SNc DAn loss, the Lewy body-like accumulations suggest that chronic rotenone treatment can mimic PD pathology.

Rotenone Induces or Reduces Apoptosis. *In vitro*, acute rotenone treatment has been shown to induce apoptosis in a variety of different cells in culture, including tumor cells [73], PC12 cells [74, 75], myeloid cells [76], lymphoblasts [77], SK-N-MC dopaminergic cell lines [75], HL-60 cells [64, 78], AK-5 tumor cells [79], PAJU human neuroblastoma cells [80], liver cells [81], human dopaminergic neuron-like cells [82], and cerebellar granule neurons [83]. In contrast, rotenone has been found to reduce apoptosis in models like 1-O-octadecyl-2-methyl- rac-glycero-3-phosphocholine in p53-defective hepatocytes [84], glutamate in rat hippocampal neurons [85], hypoxia in cardiac myocytes [86] and protein kinase C overexpressing keratinocytes [87]. In short, rotenone can either increase or decrease apoptosis, depending on cell type and the insult that initiates the apoptosis.

Rotenone Reduces, Rather Than Increases, ROS Generation *In Vitro.* Imaging studies of cultured neurons, including DAns [33, 34] or neuron-like cells [88] utilized dyes whose fluorescence increases in proportion to the concentrations of superoxide or peroxyl radicals. Those studies showed that decreased mtRCx I activity caused by rotenone is accompanied by a decrease, rather than an increase, in ROS levels [33, 88, 89]. Like those studies, we have also found that acute rotenone treatment of cultured CAergic neuron-like cells decreases peroxyl levels and does not alter superoxide levels in the somata of the cells (see below). Therefore, decreased mtRCxI activity may not induce neuronal loss through ROS peroxidation of macromolecules which was previously supposed to form the basis for neuronal death caused by decreased mtRCx I activity [90, 91].

Rotenone, Mitochondrial Membrane Permeability, Mitochondrial Membrane Potential ($\Delta\Psi_M$) And The Mitochondrial Permeability Transition Pore Complex (PTPC). Mitochondria can play a role in either apoptotic or necrotic cell death [92]. Cytochrome c, AIF and smac/diablo are constituents of the mitochondrial intermembrane space. Once released into the cytosol, cytochrome c binds to Apaf-1 and leads to the activation of caspases, particularly caspase 3. Smac/diablo counteracts inhibitor of apoptosis proteins (IAP) capacity to prevent caspase activation [93-95], while AIF activates nuclear endonucleases [96, 97]. The mechanisms which increase permeability of the outer mitochondrial membrane and allow the release of the three proteins are incompletely understood. Two processes have been implicated: 1) the formation of channels in the outer membrane and 2) mitochondrial swelling due to opening of the PTPC causing outer membrane rupture [98, 99] .

A number of studies have used cyclosporin A (CSA), N-methyl-4-valine cyclosporin (N-Val-CSA) or bongkrekic acid (BA), each of which facilitates PTPC closure, to determine whether the PTPC opening contributes to cell death caused by rotenone. Studies using CSA in rotenone treated cells suggested that apoptosis due to mtRCx I inhibition depended on PTPC opening [75, 81, 100]. The PTPC closing agents decrease apoptosis and prevent decreases in $\Delta\Psi_M$ that are thought to result from dissipation of the transmembrane proton gradient caused by PTPC opening (see [92, 101, 102] for

reviews). Similar to studies in other cell types [103-106], we have used CSA and BA in CAergic neuron-like cells to reduce apoptosis and prevent $\Delta\Psi_M$ dissipation caused by rotenone (see below). BA was particularly effective at concentrations as low as 10^{-9} M.

GAPDH In Neurodegeneration And Apoptosis – GAPDH was found to co-immunoprecipitate with the mutant proteins in Huntington's disease (HD) and related degenerative conditions [107]. It was hypothesized that GAPDH binding to the mutant proteins might impair glycolysis [108] but that possibility has not been supported by glycolytic measurements in postmortem brain tissue and cultured cells [54, 109, 110].

Studies with antisense oligonucleotides showed that GAPDH is essential to the progression of apoptosis involving a variety of neuronal cells [111, 112]. GAPDH mRNA and protein levels increase in neurons early in apoptosis caused by reduction of media K^+ [113, 114], cytosine arabinoside exposure [112] and aging [111]. We found that GAPDH levels begin to increase at 1.5-2.0 hours after serum and NGF withdrawal, which is 4 hours before nuclear DNA cleavage and chromatin condensation began to appear in the same cells [54]. Studies with antisense oligonucleotides have shown that p53 is upstream to GAPDH increases in apoptosis [115].

In non-apoptotic cells, GAPDH is primarily in the extra-nuclear cytoplasm with sparse localization to small punctate areas in the nucleus [116]. In apoptosis involving GAPDH upregulation, GAPDH accumulates densely in the nucleus [54, 117-119], which serves as a marker of GAPDH associated apoptosis.

We cloned a GAPDH cDNA from rat brain and used the cDNA to produce a GAPDH-green fluorescent protein (GFP) construct [120], which was transiently transfected transiently into COS1 cells and stably transfected into HEK 293 cells. The living cells had little or no GAPDH-GFP protein in their nuclei and showed a distribution for the fusion protein that was largely cytosolic. The GAPDH-GFP fusion protein progressively accumulated in the nucleus of a proportion of the cells in the first two hours after exposure to apoptosis initiating agents, which established that GAPDH movement from the cytosol to nucleus occurs dynamically during the early stages of apoptosis. Our western blots for the nuclear subcellular fraction have shown progressive increases in nuclear GAPDH immunoreaction over the first 3-6 hours of some forms of apoptosis [54]. Ischitani et al. have used antibodies that are specific for GAPDH tetramer or for GAPDH dimer/monomer to show that the dimer/monomer form rather than the tetramer form translates to the nucleus in apoptosis (personal communication).

We recently showed that GAPDH co-localizes and co-immunoprecipitates with promyelocytic leukemia (PML) protein [116]. The co-localization and co-immunoprecipitation depended on an association of the proteins with RNA. PML protein is pro-apoptotic and is involved with nuclear proteins that control translation [121-125]. Our finding is in keeping with previous work suggesting that GAPDH modulates transcription or translation [126].

Deprenyl-Related Propargylamines Target GAPDH To Reduce Apoptosis. (-)-Deprenyl, an MAO-B inhibitor, was first used in PD together with levodopa to reduce dopamine metabolism and increase striatal dopamine availability [127, 128]. Studies of MPTP toxicity in the monkey [129] were interpreted to show that (-)-deprenyl reduced dopaminergic neuronal necrosis caused by ROS generated by dopamine metabolism. The finding fostered clinical trials examining the effect of (-)-deprenyl on the clinical progression of PD (see [130-134] for examples). The trials provided clear evidence that (-)-deprenyl slows the clinical progression of PD. There has not been agreement on the mechanisms responsible for the slowing (see [132, 135-137]), particularly whether the results indicate slowing of neuronal death in the disease.

Although it is uncertain whether (-)-deprenyl slows the progressive neuronal death in PD, it does reduce neuronal death in a wide variety of *in vivo* and *in vitro* experimental models. The models have included neuronal death induced by 6-hydroxydopamine [138], MPP^+ [139-146]; MPTP [129, 144, 147], nitric oxide or peroxynitrite [148], DSP-4 [149-151]; glutathione depletion [152], peripheral nerve crush or axotomy [153-157]; optic nerve crush [158]; hypoxia and/or ischemia [159] [160-163]; cytosine arabinoside [164]; excitotoxins [165] [163, 166, 167], trophic insufficiency [54, 55, 168, 169]; thiamine deficiency [170] and aging [171-174].

A number of investigations have established that (-)-deprenyl can reduce neuronal death independently of MAO-B or MAO-A inhibition [141-144, 149, 153, 165, 175, 176]. Studies in a number of models have shown that (-)-deprenyl can reduce apoptosis [55, 80, 141, 148, 159, 164, 165, 168, 169, 177-189]. The reduction of apoptosis by (-)-deprenyl has been found to involve prevention of decreases in mitochondrial membrane potential [55, 164], which may indicate maintenance of mitochondrial membrane impermeability.

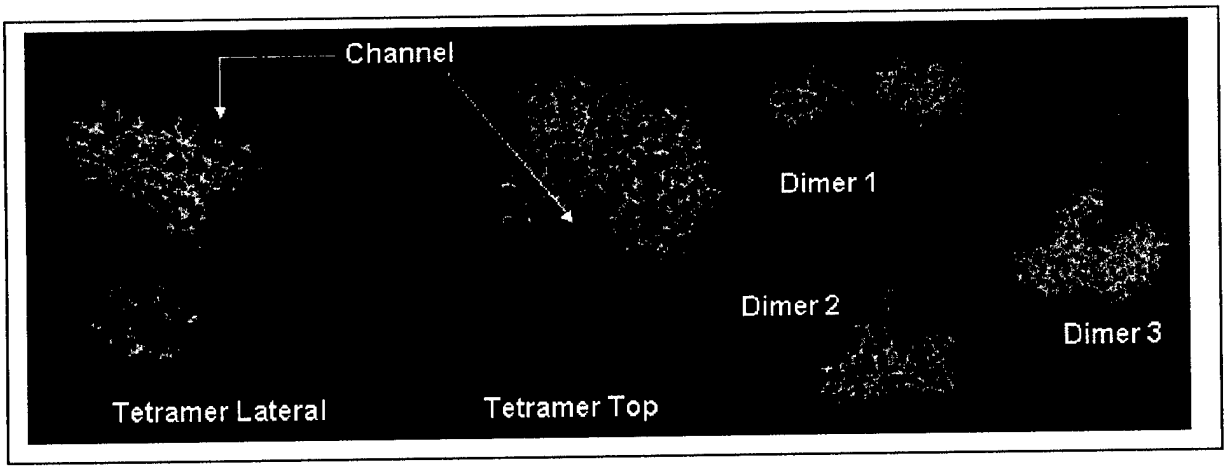


Figure 1 – Conversion of GAPDH Tetramer To A Dimer By CGP3466 In Solution. Our use of Size exclusion chromatography showed that the tricyclic deprenyl analog, CGP3466 and other propargylamines convert GAPDH tetramer to a dimer in solution [54]. The figure shows a computer generated model of GAPDH three dimensional structure based on yeast crystal structure with substitution of human amino acid differences (see higher resolution images and the details of our image generation in [54]). Our studies indicated that CGP3466 binds into the channel of GAPDH in order to convert the tetramer to dimer, likely dimer 3 shown in the figure and stabilizes the protein in that form. Studies by Ishitani and coworkers (Ishitani, personal communication) have shown that GAPDH translocates to the nucleus in apoptosis as a monomer or as dimer 1 or dimer 2. Antibodies against dimers 1 or 2 or against GAPDH monomer but not GAPDH tetramer block MPP+ induced apoptosis in cultured dopaminergic neurons [190].

Together with workers at Novartis, we developed a tri-cyclic deprenyl analog, CGP3466 [191], which reduces a number of forms of apoptosis in a variety of models, but does not inhibit MAO-B [192]. Photoaffinity labeled CGP3466 [193] established that CGP3466 binds to GAPDH in rat hippocampus [80] and cultured catecholaminergic cells [54]. (-)-Deprenyl and CGP3466 reduce apoptosis over similar concentration ranges (10^{-5} to 10^{-11} M) [54, 80]. CGP3466 prevented the GAPDH upregulation and the dense GAPDH nuclear accumulation typical of GAPDH-associated apoptosis [54]. We used size exclusion chromatography to show that the CGP3466 converted GAPDH from a tetramer to a specific dimer in solution ([54] and figure 1). Our findings suggest that CGP3466 binding to GAPDH prevents apoptosis signaling by the protein, while allowing it to retain glycolytic capacity.

We initially showed anti-apoptosis by (-)-deprenyl requires new protein synthesis [168]. It was not known how changes in new protein synthesis might cause the reduced apoptosis induced by propargylamines like CGP3466. We have now used metabolic labeling and western blotting to show that the propargylamines induce marked changes in new protein synthesis, particularly in the nuclear and mitochondrial protein fractions, and that those changes affect apoptosis related proteins (see below). It remains to be determined how propargylamine binding to GAPDH might induce anti-apoptotic new protein synthesis.

Report Of Progress

Progress In Ascertaining Whether GAPDH Contributes To Apoptosis In A Decreased mtRCx I Activity Model Of PD - In our previous progress report, we presented our findings from work aimed at investigating mechanisms of GAPDH and mitochondria in apoptosis signaling. Although MPTP has provided the standard model of PD over the last 20 years, a number of recent findings indicate that the capacity of MPTP to model decreased mtRCx I activity PD is limited. In contrast, rotenone may offer a superior PD model from the aspect of decreased mtRCx I activity. Studies of postmortem brain suggest that GAPDH signaling may be implicated in PD neuronal apoptosis. Accordingly, we sought to determine whether rotenone models the GAPDH-related aspects of PD.

Rotenone Induced Apoptosis Requires New Protein Synthesis, Including A Pathway Involving Increased GAPDH Levels And Dense GAPDH Nuclear Translocation. Some forms of apoptosis require the new synthesis of proteins, while other forms do not (see discussion in [194], appended). We used actinomycin D at 2 μg/ml and cycloheximide at 8 μg/ml to inhibit transcription and translation respectively in NGF differentiated PC12 cells, a CAergic neuron-like cell line. The cells were exposed to varying rotenone concentrations. In other studies, we had showed that those concentrations of actinomycin D and cycloheximide did not decrease survival of the serum and NGF supported CAergic cells but did decrease levels newly metabolically labeled proteins in the cells by 94% or greater ([194], appended). The protein synthesis inhibition revealed that about two-thirds or more of the decreased survival induced by rotenone required new protein synthesis (figure 2) and established that new protein synthesis is required for rotenone induced apoptosis in the cells

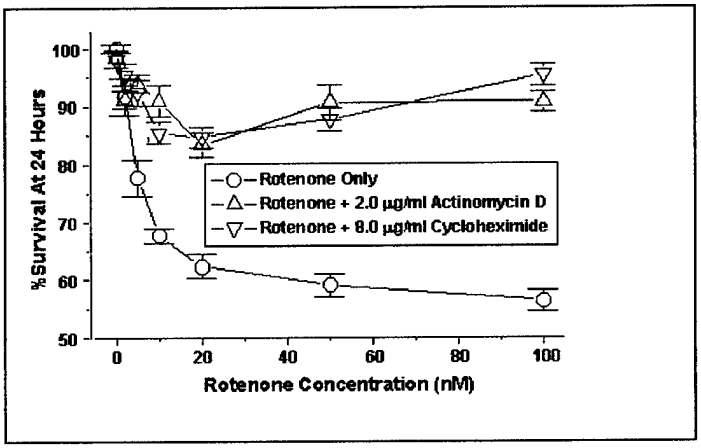


Figure 2 –Transcriptional Inhibition And Translational Inhibition Reduce The Loss Of Catecholaminergic Neuron-Like Cells Induced By Rotenone. Actinomycin D and cycloheximide were utilized to determine whether apoptosis induced by rotenone depends on new protein synthesis . Both inhibitors increased cell survival indicating that a major proportion of the decreased survival induced by rotenone depends on new protein synthesis in the cultured cells. The proteins that are newly synthesized in rotenone induced apoptosis are not known. It is also not known whether or how decreased mtRCxI activity induces the new protein synthesis.

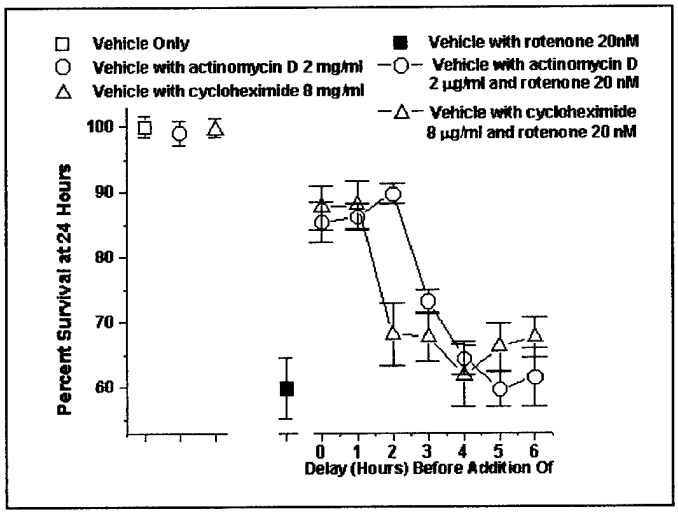


Figure 3 – Delayed Application Of Transcriptional And Translational Inhibitors Reveals the Timing Of Expression Of Essential Apoptotic Proteins. Experiments in which the application of the transcriptional and the translational inhibitor were delayed relative to the addition of rotenone to the cultured catecholaminergic cells showed that the genes for proteins that are essential to decreasing survival were translated between 0 and 3 hours after rotenone addition. Our western blots have shown that GAPDH levels begin to increase between 2.5 and 3.0 hours after rotenone addition to the cultured cells

Experiments in which the application of new protein synthesis inhibitors was delayed relative to the addition of rotenone indicated that pro-apoptotic new protein synthesis induced by rotenone was largely transcribed and translated

between 1 and 4 hours after rotenone addition (figure 3, also see [194] appended for details of the methods and interpretation of the delay experiments). GAPDH has been shown to upregulate in a number of forms of neuronal apoptosis and antisense oligonucleotide application or intracellular antibody injection has shown that increased synthesis of GAPDH is essential to the neuronal apoptosis [54, 111, 113, 114], including apoptosis induced by MPP^+ in primary cultures of dopaminergic mesencephalic neurons [190]. Accordingly, GAPDH synthesis is critical to some forms of apoptosis. We previously found that GAPDH levels increased at 1.5 to 2 hours after serum and NGF withdrawal from the CAergic cells [54]. In the present experiments, We found that GAPDH levels increase in the CAergic cells after exposure to rotenone. GAPDH levels were found to be increased by 3 hours after rotenone application and the GAPDH immunodensity varied with rotenone concentration for concentrations of 2 - 20 nM according to a U shaped relationship with the maximum increase for 10 nM (figure 4).

An identifying feature of apoptosis involving GAPDH signaling is the dense nuclear accumulation of GAPDH immunoreaction [54, 117-119], due to GAPDH translocation from the cytosol to the nucleus [120]. GAPDH is normally sparsely distributed in the nucleus in association with promyelocytic leukemia (PML) protein [116]. Since dense accumulation GAPDH nuclear immunoreaction has been found in neuromelanin containing neurons of the PD SNc [14], we examined the CAergic neuron-like cells acute rotenone exposure for GAPDH dense nuclear accumulation.

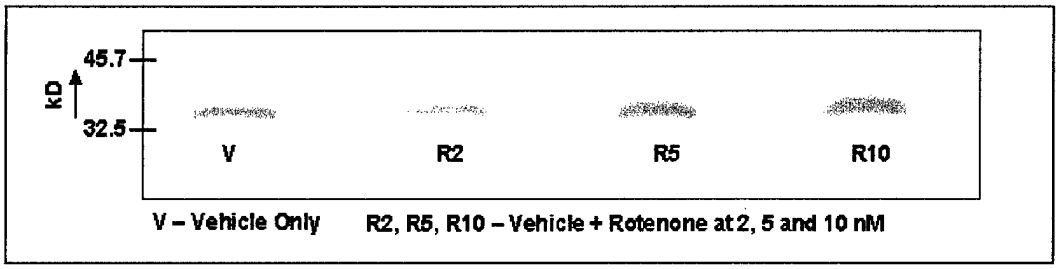


Figure 4 - Rotenone Increases GAPDH Levels In A Concentration Dependent Manner. Western blots showed that rotenone concentrations which induced decreases in mtRCxI activity of 15-45%, also increased GAPDH levels in a concentration related fashion beginning at 2-3 hours after the rotenone addition (this blot is for 6 hours after rotenone addition).

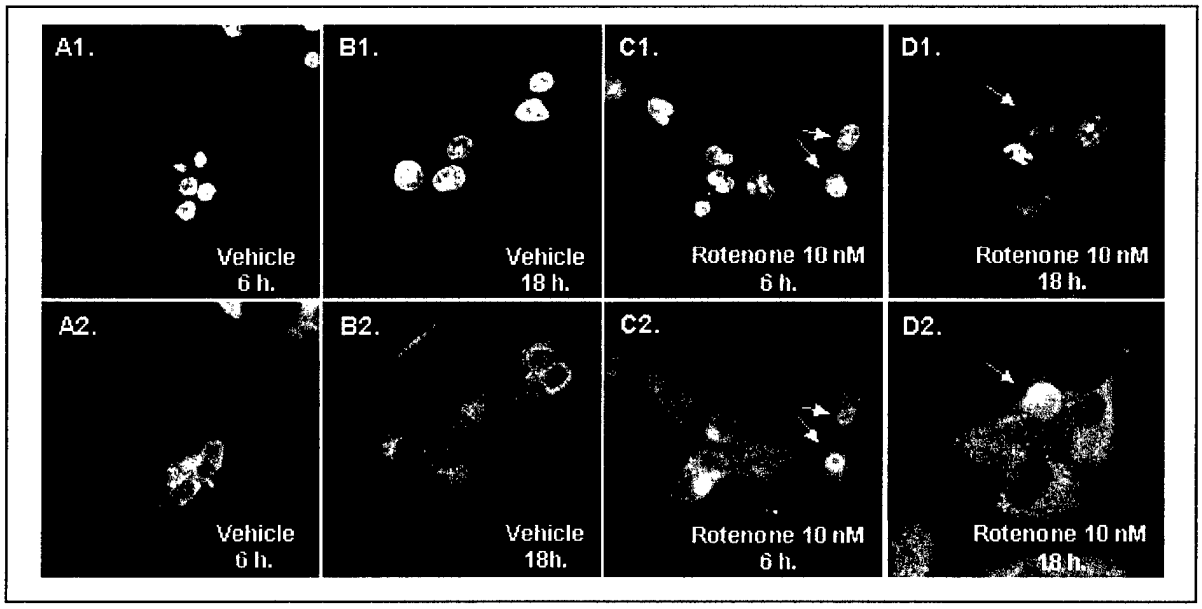


Figure 5 - Rotenone Induces Accumulation Of Dense Nuclear GAPDH Immunoreaction - Rotenone induced GAPDH dense nuclear immunoreaction typical of GAPDH signaling in apoptosis. Vertical pairs of LCSM images are for identical image fields. Upper images are for YOYO-1 staining of DNA and lower images for GAPDH immunofluorescence. A1,A2 and B1,B2 are for vehicle treated cells and C1,C2 and D1,D2 for rotenone treated cells. The arrows denote nuclei with dense nuclear GAPDH immunoreaction.

As shown in figure 5, rotenone exposure of the CAergic cells induced dense nuclear GAPDH immunofluorescence in a proportion of the cells and the dense nuclear accumulation was not present in vehicle treated cells (compare figure 5 A1/A2 and B1/B2 to C1/C2 and D1/D2). Accordingly, decreased mtRCx I activity caused by rotenone exposure induces GAPDH upregulation and dense nuclear GAPDH accumulation similar to that found in SNc neuromelanin containing neurons in PD postmortem brain.

Chronic Rotenone Exposure Induces Ubiquitin Immunopositive Aggregations In The Cell Bodies Of Cultured Catecholaminergic Cells. DAergic neurons in the rat SNc have shown protein aggregations with similarity to Lewy bodies found in PD after chronic intravenous rotenone exposure [72]. This contrasts to MPTP or MPP+ treated cells which do not show PD-like protein accumulations. We treated NGF differentiated CAergic neuron-like cells for 8 to 21 days with 1 to 2 nM of rotenone. The rotenone containing media was replaced daily. About 17% of cells surviving for 14 to 21 days showed patchy regions of intense ubiquitin immunofluorescence (see figure 6). Although ubiquitin is a marker of Lewy bodies [12], α-synuclein has become to be considered as the major component of Lewy bodies [13, 195] and it has been shown that α-synuclein aggregations and even fully formed Lewy bodies are prominent constituents of axons and terminals of CAergic neurons in PD and several related conditions [196]. Our preliminary findings showed that α-synuclein immunofluorescence co-localized with the patchy ubiquitin immunofluorescence in cell bodies. While ubiquitin and α-synuclein were localized to the peripheral portion of the patches, GAPDH immunofluorescence localized to the central portion . In PD Lewy bodies, the three proteins are found with a similar distribution - ubiquitin and α-synuclein in the peripheral or radial portion of the bodies and GAPDH in the central portion [14].

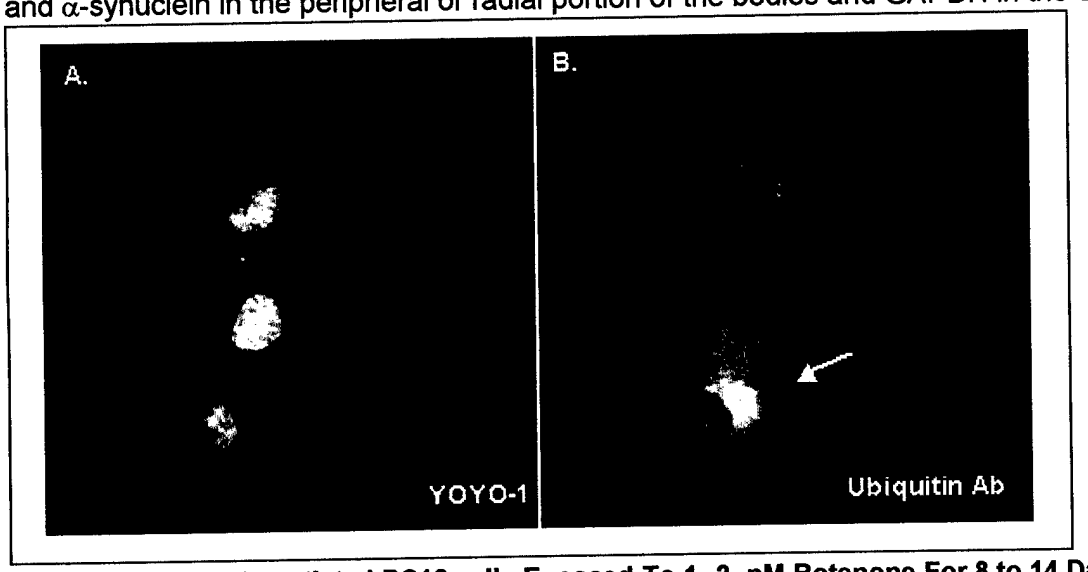


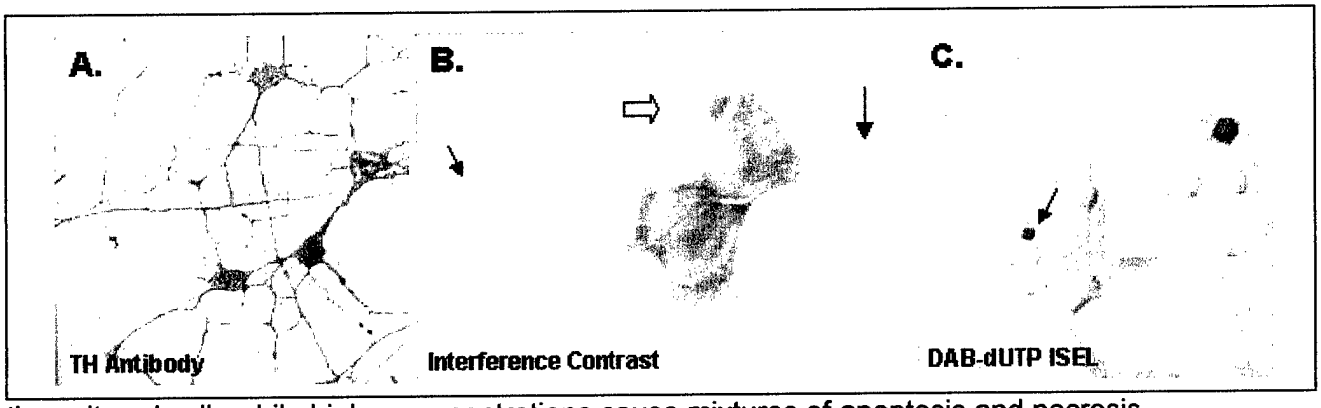
Figure 6. NGF Differentiated PC12 cells Exposed To 1 -2 nM Rotenone For 8 to 14 Days Show Cell Body Ubiquitin Immunopositive Accumulations With LCSM A. and B. are for identical image fields. A. shows YOYO-1 fluorescence for nuclear DNA while shows ubiquitin immunofluorescence. Approximately 17% of the surviving CAergic neuron-like cells showed ubiquitin immunopositive cell body accumulations similar to that in B.

Progress In Determining The Mechanisms Of Neuronal Loss Caused By Decreased mtRCx I Activity.

Decreased mtRCxI Activity Caused By Rotenone Is Associated With Process Withdrawal And Apoptosis Of Cultured Catecholaminergic Cells. We examined the effects of acute rotenone treatment on NGF differentiated PC12 cells. As illustrated in figure 7A, the cells are catecholaminergic as shown by their tyrosine hydroxylase (TH) immunoreactivity. Single applications of rotenone (5 to 20 nM) induced process truncation (solid arrows in figure 7B) with cytosolic and nuclear shrinkage of a proportion of the cells (open arrow in figure 7B). ISEL showed that the shrunken nuclei contained

fragmented DNA typical of apoptotic degradation (figure 7C).

Rotenone concentrations of 5 to 20 nM induced 32 and 38% decreases in survival respectively (figure 8A) and mean decreases in mtRCxl activity of 16 to 60 % (figure 8B). Concentrations of 10 and 20 nM induced the maximum percentages of cells with nuclear chromatin condensation (figure 8E, typical chromatin condensation associated with cell/nuclear shrinkage is illustrated in 8C1/C2 and 8D1/D2). These data suggest that 10 nM is the most effective concentration for inducing apoptosis in



the cultured cells while higher concentrations cause mixtures of apoptosis and necrosis..

Figure 7 – Rotenone Induced Process Damage And Apoptosis In CAergic PC12 Cells - A. shows immunoreaction for an antibody against TH in NGF differentiated PC12 cells. **B.** Higher power interference contrast LCSM image of rotenone treated NGF differentiated PC12 cells showing a shrunken cell with a condensed nucleus (marked by open arrow) and two other cells with normal cell bodies and nuclear morphology but truncation of their processes (shown by small arrows). **C.** dUTP in situ DNA end labeling demonstrating nuclear DNA fragmentation typical of apoptotic nuclear degradation in a shrunken NGF differentiated PC12 cell (marked by an arrow and shown at higher magnification in the inset image).

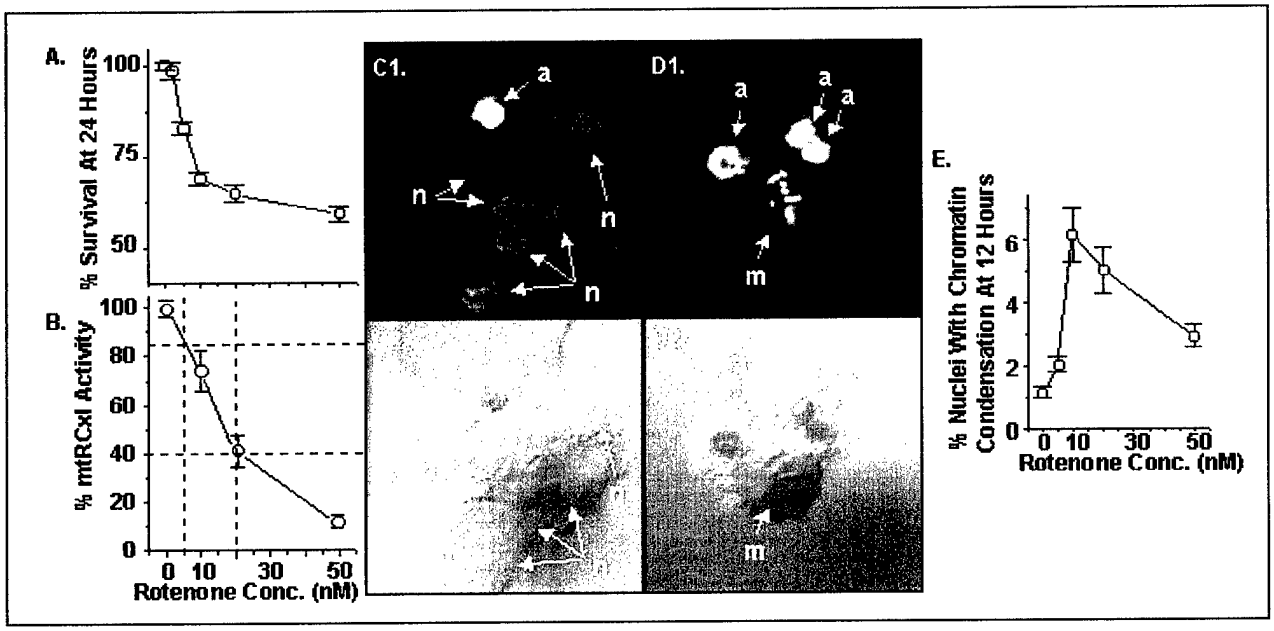


Figure 8 – Rotenone Reduces Survival, Induces Apoptosis And Decreases mtRCxl Activity In NGF Differentiated, Catecholaminergic PC12 Cells In Culture. A. shows the % survival of the cells at 24 hours after the application of different rotenone concentrations while B. shows the % mtRCxl activity versus rotenone concentration for the mitochondrial fraction of cell lysates at 3 hours after rotenone addition. The C1/C2 and D1/D2 pairs are for identical laser confocal image fields. The upper images of each pair are for the fluorescence of the nucleic acid binding dye YOYO-1 and the lower images are interference contrast images. The images show typical nuclei with chromatin condensation (labeled a),

normal nuclei (labeled n) and one nucleus in mitosis (labeled m), each at 12 hours after treatment with 10 nM rotenone. E. shows the relationship between the % nuclei with chromatin condensation versus rotenone concentration at 12 hours after treatment with 10 nM rotenone. Rotenone concentrations of 5 to 20 nM induced 32 and 38% decreases in survival respectively (figure 3A) and mean decreases in mtRCxI activity of about 16 to 60 % (figure 3B). Concentrations of 10 and 20 nM induced the maximum percentages of cells with nuclear chromatin condensation (figure 3E, typical chromatin condensation associated with cell/nuclear shrinkage is illustrated in 3C1, C2 and 3D1, D2). These data suggest that 10 nM is the most effective concentration for inducing apoptosis in the cultured cells while higher concentrations increasingly induce necrosis.

Loss Of CAergic Cells Induced By Rotenone In Culture Is Caspase Dependent But Is Energy Independent And Can Be Modulated By The Availability Of Glycolytic ATP

Previous research has shown that apoptosis induced by rotenone in AK-5 cells is caspase dependent [79]. We found that rotenone increased levels of activated caspase 3 in cells with evidence of nuclear chromatin condensation after rotenone exposure (figure 9 A1 and A2). The irreversible general caspase inhibitor ZVAD-FMK at concentrations of 10 μM induced maximum reversal of the decreased survival induced by rotenone (figure 9B). An average of approximately two-thirds of the decreased survival were reversed at different rotenone concentrations, suggesting that a major portion of the cellular loss induced by rotenone was caspase dependent. Accordingly, GAPDH associated apoptosis induced by rotenone is caspase dependent, particularly caspase 3 dependent, which is similar to the apoptosis found in neuromelanin containing neurons in the SNc of PD postmortem brain (see references above).

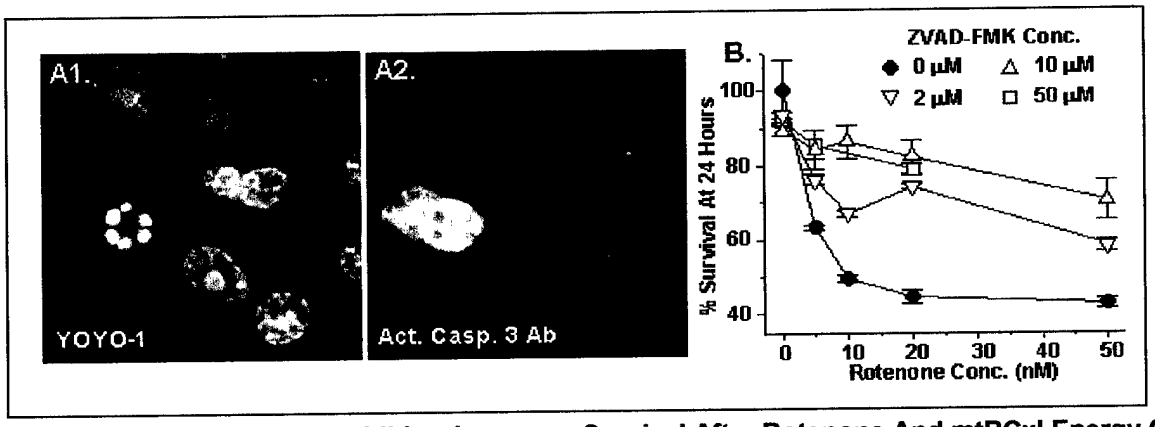


Figure 9 – General Caspase Inhibitor Increases Survival After Rotenone And mtRCxI Energy Or Glucose Dependence Of Rotenone Induced Neuronal Death. A1. and A2. show identical LCSM image fields. YOYO-1 staining shows one nucleus with chromatin condensation in a number of nuclear bodies. Other nuclei show normal staining. The cell with nuclear chromatin condensation shows high levels of activated caspase 3 immunofluorescence. **B.** ZVAD-FMK at varying concentrations was delivered to the cells 30 minutes prior to rotenone addition. ZVAD-FMK is an irreversible, cell permeant caspase inhibitor.

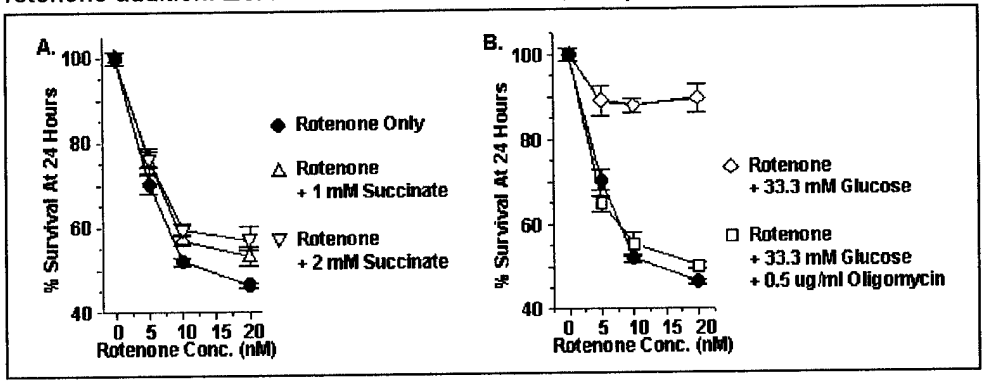


Figure 10 –Mitochondrial Energy Independence And Glycolytic Energy Dependence Of Rotenone Induced Neuronal Death. A. High concentrations of succinate provide energy to mitochondrial complex II, bypassing energy decreases induced by rotenone by inhibiting mtRCxI. The plots show that less than 10% of the death is mtRCxI energy dependent for rotenone concentrations of 10 nm or less, concentrations, which largely induce apoptosis (see above). About 15% of the death caused by 20 nM rotenone is mtRCxI energy dependent.

B. Increased glycolytic ATP produced by high glucose concentrations is used by ATP synthase to reverse pump protons outward across the inner mitochondrial membrane and can reduce decreases in $\Delta\Psi_M$ caused by mtRCxI inhibition (see below and [198]). We found that high glucose concentrations in the supporting media reverses 75% or more of the cell loss induced by rotenone and that oligomycin, an ATP synthase inhibitor, blocks the compensation offered by high glucose concentrations.

We used varying concentrations of succinate, the substrate for mitochondrial respiratory complex II [197], in order to assess the role that decreased provision of energy by mtRCxI played in the reduced survival induced by rotenone (figure 10A). The experiments revealed that increasing energy provision to complexes II - IV induced relatively small increases in survival and therefore showed that a reduction in energy provision by mtRCx I played little role in rotenone induced apoptosis in the differentiated PC12 cells. Deficient energy availability has been a central tenet of the hypothesized mechanisms of PD neuronal loss caused by decreased mtRCx I activity and now must be re-evaluated.

A second series of experiments provided high glucose concentrations to the cells as a means of increasing the availability of glycolytic ATP (see [198] for our similar experiments with MPP^+). The experiments showed that high glucose largely reversed the decreased survival caused by rotenone (figure 10B) and that oligomycin, a selective inhibitor of mitochondrial ATP synthase, almost completely eliminated the prevention of cell loss provided by high glucose. A number of studies have shown that ATP synthase can use glycolytic ATP to pump protons outward across the inner mitochondrial membrane and thereby maintain $\Delta\Psi_M$ (see [198] for details). mtRCx I as well as complexes III and IV normally use energy extracted from NADH, reduced ubiquinone and cytochrome C to pump protons outward across the inner mitochondrial membrane and the resulting transmembrane proton gradient contributes to the generation of $\Delta\Psi_M$. Accordingly, it appears that reverse proton pumping by ATP synthase can compensate for neuronal death caused by mtRCx I inhibition

Rotenone Does Not Increase ROS Levels In Cell Bodies Of CAergic Neuron-Like Cells. We used the dyes 2'-7'-dichlorofluorescein ($DCFH_2$-DA) [199-201] and dihydroethidium (HEth) [202-204] to estimate peroxyl radical and superoxide radical levels in the cell bodies of the CAergic cells after rotenone treatment (see figure 11 for typical LCSM images for the two dyes).

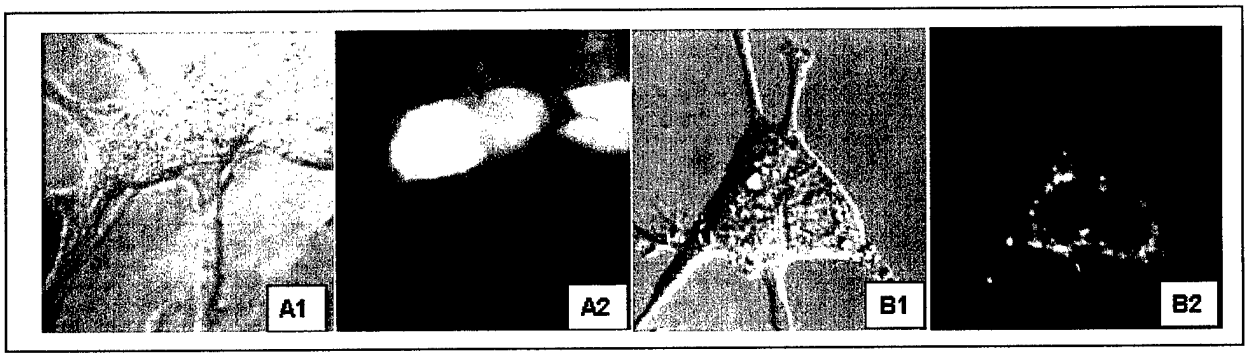


Figure11 - $DCFH_2$-DA and Dihydroethidium LCSM Images. A1 and B1 are LCSM interference contrast images while **A2 and B2** present 2', 7'-dichlorofluorescein and ethidium ion fluorescence in the cell bodies of living NGF differentiated PC12 cells. The images were taken at maximum gain to display the localization of the ROS fluorescence. Note the cell to cell variation in peroxyl radical induced fluorescence shown by DCF in A2 and the punctate nature of superoxide radical induced fluorescence in B2. The cells were treated with 10 nM rotenone 3 hours prior to the imaging.

Loss of mitochondrial energy due to decreased mtRCx I activity, which in turn fosters increased ROS levels leading to neuronal death, has been a central hypothesis for the basis of neuronal loss in PD. Our findings suggest that decreased mtRCx I activity is not necessarily accompanied by either of those defects. Accordingly, decreased mtRCx I activity may induce neuronal apoptosis in PD through other means.

Rotenone Decreases $\Delta\Psi_M$ In Cell Body Mitochondria Of Catecholaminergic Cells And Opening Of The Mitochondrial Permeability Transition Pore Contributes To Rotenone Induced Apoptosis. Decreases in $\Delta\Psi_M$ accompany a number of forms of apoptosis and likely result from increases in the permeability of the inner mitochondrial membrane with dissipation of the transmembrane protein gradient. As shown in figure 13, we found that rotenone induced a concentration dependent decrease in $\Delta\Psi_M$ and oligomycin caused a further decrease in $\Delta\Psi_M$. This data (also see above) suggest that ATP synthase partially compensates for caused by rotenone, likely by pumping protons out across the inner membrane using energy provided by glycolytic ATP. In other experiments, succinate was added as in the survival experiments shown above (data not shown) and revealed that provision of substrate to complex II did not significantly alter the rotenone-$\Delta\Psi_M$ relationships. This suggested that a reduction in proton pumping by mtRCxI is unlikely to be a major factor in the generation of the decreased $\Delta\Psi_M$ caused by rotenone induced decreases in mtRCx I activity.

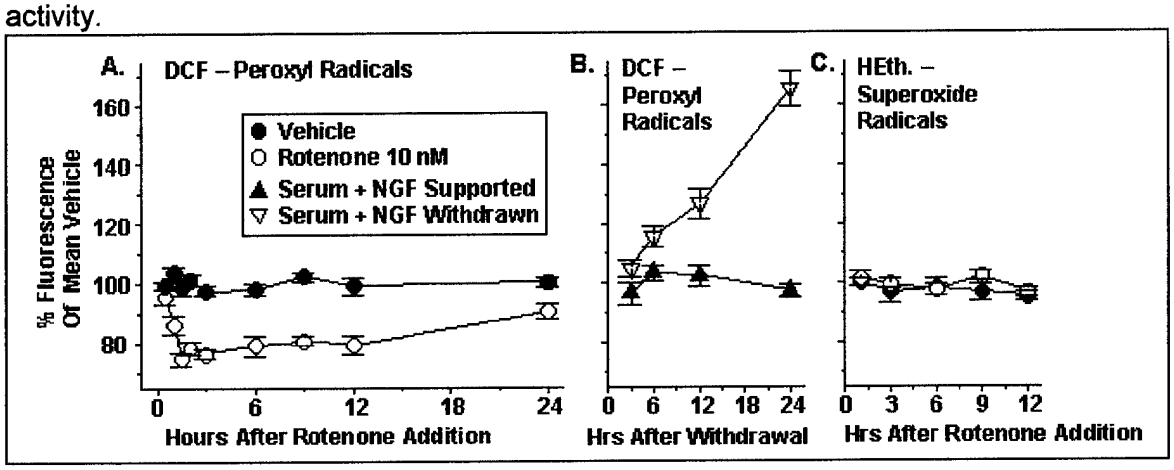


Figure 12 - Rotenone Decreases Peroxyl Radicals And Does Not Alter Superoxide Radical Levels In The Cell Bodies Of Living Catecholaminergic Neuron-Like Cells - A. Peroxyl radical levels estimated from LCSM images of living cells that compared vehicle treated to 10 nM rotenone treated cells at time points varying from 30 minutes to 24 hours after treatment. B. Peroxyl radical levels estimated by $DCFH_2$-DA induced florescence compared in serum and NGF supported versus serum and NGF withdrawn cells at multiple time points (see [55] for more details). C. Superoxide radical levels shown by HEth. florescence comparing cells treated with 10 nM rotenone or vehicle. All points are for 200 to 400 cell body measurements in living cells.

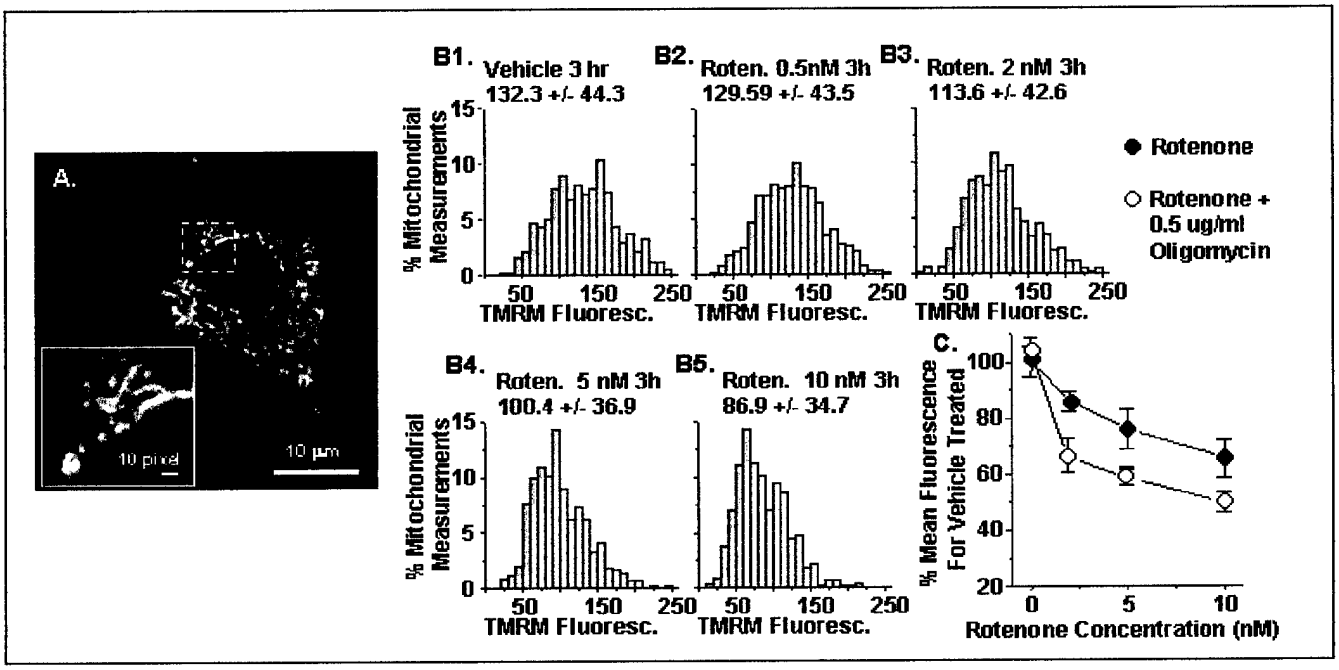


Figure 13 - Measurement Of $\Delta\Psi_M$ In Live Cells Exposed To Varying Concentrations Of Rotenone. We used three different mitochondrial potentiometric dyes to measure $\Delta\Psi_M$ by imaging dye fluorescence with LCSM (see [55] appended for details). A. Typical image for Tetramethylrhodamine methyl ester (TMRM) fluorescence is shown for single NGF differentiated PC12 cells. The inset is included to demonstrate the capacity to resolve single mitochondrial profiles. **B1. - B5.** TMRM, used in living cells, revealed a rotenone concentration dependent shift of the distributions found for mitochondrial fluorescence toward lower values (each distribution represents 600 - 900 measurements) that was evident as early as 30 minutes after rotenone addition. The decreases remained unchanged after 3 hours. **C.** Addition of oligomycin with rotenone increased the shifts toward lower values. Oligomycin is an inhibitor of ATP synthase and blocks glycolytic ATP proton pumping by ATP synthase that partially compensates for $\Delta\Psi_M$ dissipation.

Several studies have shown that agents, which facilitate closure of the PTPC, can reduce cell death caused by rotenone [75, 81, 100]. We found that BA, which binds to the adenine nucleotide translocator (ANT), a major component of the PTPC, and cyclosporine A, which prevents cyclophilin D binding to ANT , both increased the survival of rotenone treated cells (figure15A). BA was most effective at 10^{-9} M and was able to increase survival to a significantly greater extent than CSA (data not shown). We also used LCSM imaging of 5,5',6, 6'-tetrachloro-1,1',3,3'-tetraethybenzimidazol carbocyanine iodide (JC-1) fluorescence to estimate $\Delta\Psi_M$ in living cells (see [55] for details and figure 14 for an example). The studies showed that 10^{-9} M BA markedly attenuated the decreased $\Delta\Psi_M$ induced by rotenone (figures 7B1-7B3 and 7C1 -7C3). We interpret these findings to show that both the decrease in survival and the decrease in $\Delta\Psi_M$ caused by rotenone in the cultured Catecholaminergic cells were in large part due to opening of the PTPC.

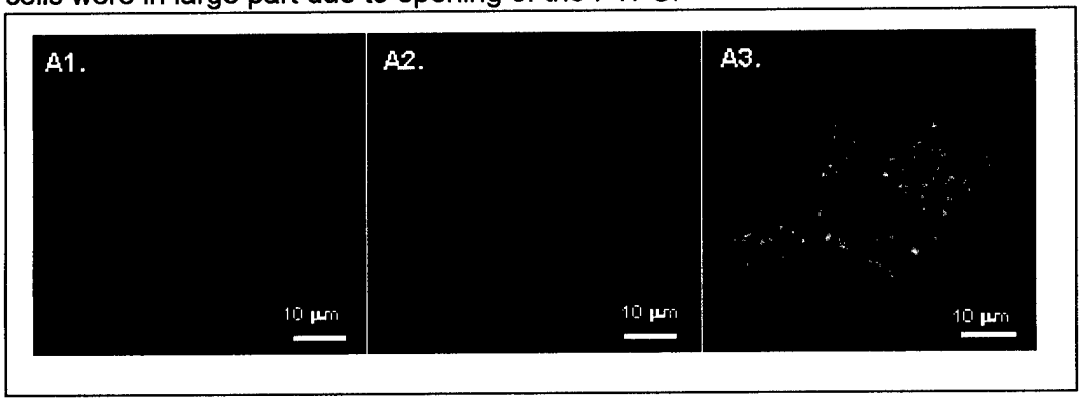


Figure 14 - Typical images for 5,5',6, 6'-tetrachloro-1,1',3,3'tetra-ethybenzimidazol carbocyanine iodide (JC-1) fluorescence to estimate $\Delta\Psi_M$ in living cells living NGF differentiated PC12 cells. The red-green added figure in C3 illustrates the range of $\Delta\Psi_M$ in different mitochondrial profiles (green indicates low $\Delta\Psi_M$, yellow indicates intermediate $\Delta\Psi_M$ and orange-red indicates high $\Delta\Psi_M$).

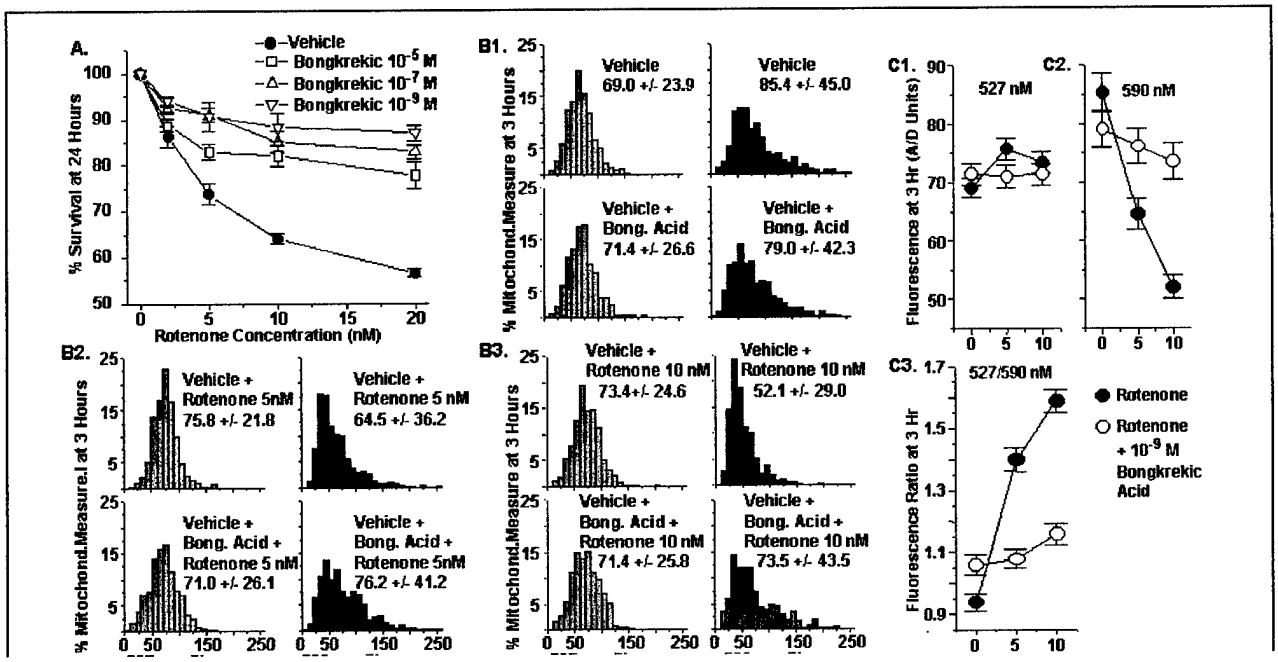


Figure 15. Bongkrekic Acid Attenuates The Decreased Survival And Cell Body $\Delta\Psi_M$ Dissipation Caused By Rotenone In Cultured Catecholaminergic Neuron-Like Cells. A. BA concentrations of 10^{-9} M were most effective in reducing the cellular loss caused by rotenone (78% or more at all concentrations of rotenone). **B1-B3.** BA shifted the distributions for the 590 nm emission of JC-1 to higher values compared to those for rotenone alone. It either decreased or maintained the values of distributions for the 527 nm JC-1 emission. **C1-C3** summarize the changes in dual emission and present the values for the 527 nm/590 nm ratio (all of the distributions are for 600 or more LCSM measurements and the plots present mean +/- SEM).These findings indicate that the majority of cell loss and the decrease in cell body $\Delta\Psi_M$ caused by rotenone depend on opening of the PTPC.

Rotenone Induces Greater Dissipation Of $\Delta\Psi_M$ In Axon Terminal Mitochondria Than In Cell Body Mitochondria. Examination of LCSM images of primary cultures of ventral mesencephalon suggested that rotenone induces relatively greater decreases in $\Delta\Psi_M$ for TH immunopositive terminals than TH immunopositive cell bodies, GABA-ergic cell bodies and terminals, and glia cells in the same cultures (figure 16, see [55] for details of our use of CMTMR to measure $\Delta\Psi_M$). Similarly , mitochondria in the terminals of CAergic neuron-like cells exposed to serum and NGF cells for 17 days to maximize axon growth and terminal development showed a similar apparent disparity in terminal and cell body $\Delta\Psi_M$ after rotenone exposure (figure 16A). We used TMRM with LCSM imaging to compare $\Delta\Psi_M$ measurements for two rotenone concentrations (2 nM and 10 nM) in the cell bodies and terminals of the 17 day differentiated cells . Those preliminary measurements revealed a considerably greater dissipation of $\Delta\Psi_M$ in the terminals compared to the cell body mitochondria. (figure 16B).

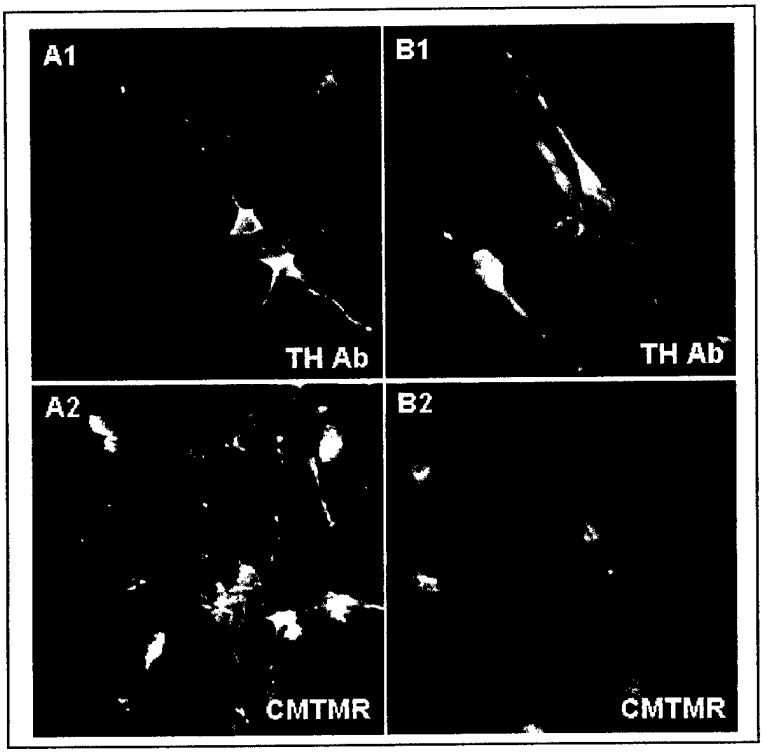


Figure 16. DAns In Primary Mesencephalic Cultures. A1 and A2 and B1 and B2 are for identical LCSM image fields. **A1 and B1** show DAn cell bodies and processes identified by TH immunofluorescence. **A2 and B2** show images of $\Delta\Psi_M$ by the potentiometric dye CMTMR. The intensity of CMTMR fluorescence estimates $\Delta\Psi_M$ if the potentiometric dye is incubated with the cells only briefly before fixation (i.e. 10 minutes, see [205] appended). Immunofluorescence for the TH antibodies showed little difference for vehicle treated (A1) and those treated with 10 nM rotenone (B1). In contrast, overall CMTMR fluorescence was greatly reduced for 10 nM rotenone treatment (B2) compared to vehicle treatment (A2). TH immunopositive cell bodies as well as neuron like non TH immunopositive cell bodies (putative GABAergic neurons) and the larger glial cells also showed decreased CMTMR fluorescence. CMTMR fluorescence can still be detected in cell bodies but is no longer detectable in TH immunopositive processes. Accordingly, the greatest decrease in CMTMR fluorescence and likely $\Delta\Psi_M$ was evident in the processes of the TH immunopositive cells.

A number of factors might account for greater $\Delta\Psi_M$ dissipation in the terminals: 1) rotenone binding to NADH dehydrogenase might be increased in the terminals; 2) rotenone might cause a greater decrease in NADH dehydrogenase activity in the terminals (decreased conversion of NADH to NAD^+); 3) a greater reduction in transmembrane proton pumping might result for a given decrease in NADH dehydrogenase activity; 4) decreased mtRCxI activity might have an increased capacity to open the PTPC in terminal mitochondria and 5) decreased mtRCxI activity might induce greater increases the levels of intramitochondrial Ca^{2+} in terminals, since a recent study has shown that chronic rotenone

treatment of a SH-SY5Y neuroblastoma cells increased Ca^{2+} in the cells and reduced $\Delta\Psi_M$ [206], or 6) increased ROS levels in terminals, but not cell bodies, might reduce $\Delta\Psi_M$. One or more of those factors could result in greater vulnerability of terminals to rotenone compared to cell bodies. For example, a decreased NADH dehydrogenase-ANT interaction would result in increased $\Delta\Psi_M$ dissipation due to greater PTPC opening (see above).

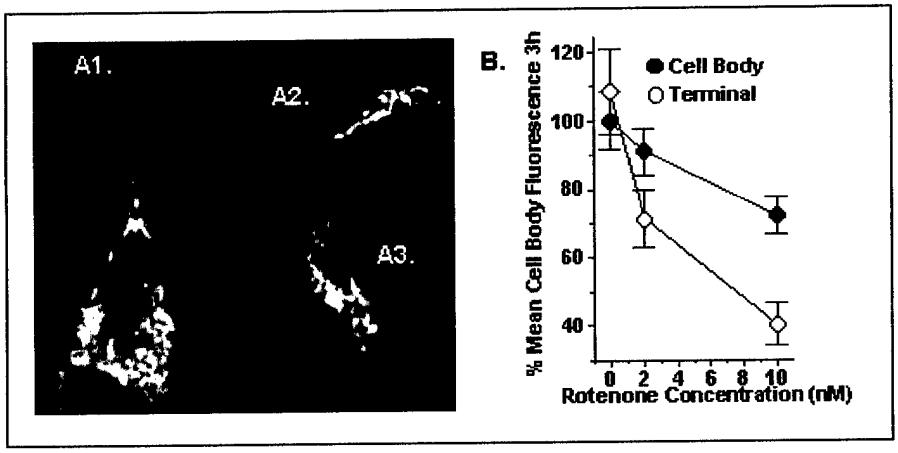


Figure 17. A1. Typical TMRM stained mitochondria in the **A1.** cell body of a CAergic neuron-like cell in culture treated with NGF for 17 days to maximize axon growth and terminal development. **A2.** and **A3.** Terminal processes of the same cell. **B.** Plots of Mean+/- SEM TMRM fluorescence of cell body mitochondria and terminal mitochondria treated with vehicle, rotenone 2 nM or 10 nM. The plots indicate that rotenone dissipates $\Delta\Psi_M$ in terminal mitochondria to a considerably greater extent compared to mitochondria in cell bodies.

Progress In Determining How Propargylamines That Bind To GAPDH Reduce Apoptosis.

Anti-Apoptotic Propargylamines Reduce Apoptosis Induced By Rotenone. The tricylic propargylamine CGP3466 does not inhibit MAO-B but binds to GAPDH and converts tetrameric GAPDH to a dimer [54, 80]. CGP3466 has been found to be extremely effective in reducing some forms of neuronal apoptosis (see [192], for a review), including that involving SNc DAergic neurons in monkeys treated with intra-carotid injections of MPTP [207]. CGP3466 is now in clinical trial as an anti-apoptotic therapy for PD (see [63] for a review of new anti-apoptotic therapies and considerations of the utility of CGP3466 in treating neurodegeneration). We are particularly pleased with the therapeutic possibilities offered by CGP3466, since it was discovered in our laboratory in 1995 [191, 192].

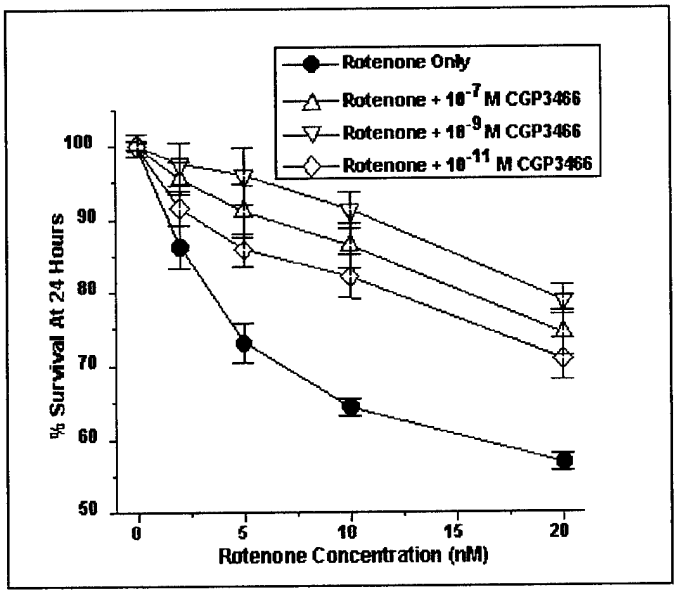


Figure 18 – The propargylamineCGP3466 Increases The Survival Of CAergic Neuron-Like Cells Exposed To The mtRCx I Inhibitor Rotenone.

We first carried out studies to determine whether CGP3466 can reduce neuronal loss induced by rotenone. As shown in figure 18, CGP3466 was most effective at nanomolar concentrations and was particularly effective in increasing the survival of the CAergic cells for rotenone concentrations in the range that induced apoptosis and caused mtRCxI activity decreases in the range reported for the PD SNc (see background material above).

Anti-Apoptotic Propargylamines Induce A Program Of Altered New Protein Synthesis That Increases Anti-Apoptotic Proteins And Decreases or Subcellularly Relocates Pro-Apoptotic Proteins. It remains to be determined how the binding of anti-apoptotic propargylamines like CGP3466 to GAPDH and the conversion of GAPDH to a dimer reduces

apoptosis, particularly apoptosis induced by decreased mtRCx I activity similar to that found in PD. In our previous progress report and our published work, we showed that the propargylamines cause down regulation of GAPDH and the prevention of dense GAPDH nuclear translocation [54]. Furthermore, our previous publications [127, 168, 208] and the rotenone research shown above suggests that the propargylamines reduce apoptosis by inducing new protein synthesis, which serves, at least in part, to maintain mitochondrial membrane impermeability [55, 164, 209] and thereby to prevent release of mitochondrial apoptosis signaling factors.

We carried out experiments in PC12 cells at various stages of NGF differentiation that were induced into apoptosis by trophic withdrawal. We used metabolic labeling with radioactive methionine combined with subcellular fractionation to show that the propargylamines alter new protein synthesis largely in the nuclear and mitochondrial subfractions. The altered new protein synthesis involved prevention of a number signaling events that promote apoptosis: 1) transient c-JUN upregulation; 2) BAX translocation to mitochondria; and 3) decreases in BCL-2, Cu/Zn superoxide dismutase (SOD1), Mn superoxide dismutase (SOD2), and glutathione peroxidase. The levels of a number of other proteins, for example cytoskeletal proteins, were unaffected by propargylamine treatment. The protein synthesis changes are appropriate to maintain impermeability of the inner mitochondrial membrane as indicated by prevention of $\Delta\Psi_M$ dissipation. They also suggest that signaling by early activating pro-apoptotic transcriptional activators like Jun-n-terminal kinase (JNK) are down regulated by propargylamine treatment. Finally, they suggest that the propargylamines maintain the ROS scavenging capacity of cells by preventing decreases in scavengers like SOD1, SOD2 and glutathione peroxidase. A number of studies have suggested that ROS play essential roles in some forms of apoptosis [210-213]. The finding are in press in the May 2002 issue of the Journal of Pharmacology and Experimental Therapeutics [194] and fully credit our Army grant for support of the research. The manuscript is appended to this progress report.

A further series of our experiments showed that exposure of the NGF differentiated CAergic cells to low, non-lethal levels of pro-oxidants induces a program of new protein synthesis that is anti-apoptotic and is very similar, if not identical, to the program induced by the anti-apoptotic propargylamines. The program maintains $\Delta\Psi_M$ like the propargylamines and alters the levels of the same spectrum of proteins as the propargylamines. is Pharmacological inhibitors of PI3-K block anti-apoptosis by low level pro-oxidant exposure and by the propargylamines. The PI3-K pathway has been shown to mediate anti-apoptosis by phosphorylating protein kinase B (Akt), which acts through a number of transcriptionally -dependent and post translational linkages to reduce apoptosis [214-216]. Accordingly, the data suggests that propargylamines activate an intrinsic pathway that protects cells from low-level damage. The finding are in press in Neuroscience [205] and fully credit our Army grant for support of the research. The manuscript is appended to this progress report.

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List of Abbreviations:

$\Delta\Psi_M$	- Mitochondrial Membrane Potential
AIF	- Apoptosis Inducing Factor
AMV	- Avian Myeloblastosis Virus
bFGF	- basic Fibroblast Growth Factor
BDNF	- Brain Derived Neurotrophic Factor
FCCP	- carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone
CMTMR	- Chloromethyl tetramethylrosamine
CC	- Chromatin Condensation
DES	- (-)-Desmethyldeprenyl
DD-PCR	- Differential Display Polymerase Chain Reaction
DMSO	- Dimethyl Sulfoxide
EDTA	- Ethylenediaminetetraacetic acid
HBSS	- Hank's Balanced Salt Solution
HSP-70	- Heat Shock Protein - 70
H2O2	- Hydrogen Peroxide
JC-1	- 5,5',6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide
LCSM	- Laser Confocal Scanning Microscopy
MEM	- Minimum Essential Medium
MPP+	- 1-methyl-4-phynelpyridinium ion
NMDA	- N-Methyl-D-Aspartate
NGF	- Nerve Growth Factor
PTPC	- Permeability Transition Pore Complex
PMSF	- Phenylmethylsulfonyl fluoride
PB	- Phosphate Buffer
PBS	- Phosphate Buffered Saline
PI3K	- Phosphatidylinositol 3-kinase
PCR	- Polymerase Chain Reaction
PC12	- Rat Pheochromocytoma Cells
ROS	- Reactive Oxygen Species
TMRM	- Tetramethylrhodamine methyl ester perchlorate
T	- Withdrawal of Serum and NGF
T_C	- Withdrawal of Serum and NGF with addition of CGP3466
T_D	- Withdrawal of Serum and NGF with addition of (-)-deprenyl

Running Title: Low-Level Pro-oxidant Exposure Reduces Apoptosis

Abstract

We found that exposure to a low-level pro-oxidant H_2O_2 (0.01 mM) reduced apoptosis in partially NGF differentiated PC12 cells that was induced 24 hours later by higher-levels of H_2O_2 (0.1 to 1.0 mM) or the withdrawal of serum and NGF. Inhibition of new protein synthesis by actinomycin D or cycloheximide showed that the anti-apoptosis by the low-level pro-oxidant pre-exposure required new protein synthesis. Studies with mitochondrial potentiometric dyes examined using laser confocal microscopy showed that the low-level pro-oxidant pre-exposure markedly attenuated the dissipation of mitochondrial membrane potential found in the cells after serum and NGF withdrawal. Western blots revealed that the low-level pro-oxidant pre-exposure prevented the decreases in BCL-2 immunodensity that was initiated in the cells in the first 6 hours after serum and NGF withdrawal. Pharmacological inhibition of phosphatidylinositol 3-kinase prevented the mediation of reductions in apoptosis by the low-level pro-oxidant pre-exposure which implicated the pathways involving phosphatidylinositol 3-kinase and protein kinase B in the reductions in apoptosis and the maintenance of mitochondrial membrane potential. Propargylamines that are structurally related to (-)-deprenyl have similarly been shown to reduce apoptosis in a wide variety of models through new protein synthesis dependent mechanisms that involve the maintenance of mitochondrial membrane potential and require the activity of phosphatidylinositol 3-kinase. We used differential display PCR to show that the changes in mRNA expression induced by the propargylamines and low-level pro-oxidant pre-exposure are very similar suggesting that they may reduce apoptosis through common mechanisms. In the present study, we have shown that pro-oxidant pre-exposure increases the survival of neuronal-like cells and that the increase in survival is due to a reduction in apoptosis. The decrease in apoptosis induced by low-level pro-oxidant pre-exposure was accompanied by a reduction in the $\Delta\Psi_M$ dissipation that is an early feature of many forms of apoptosis.

Introduction

Neuronal death is a common feature of neurological diseases. Agents that prevent damage to neurons can slow or reduce neuronal death. Neuronal death can be reduced through the activation of intrinsic mechanisms that allow neurons to resist damage [61]. Exposure to low levels of damage can activate intrinsic mechanisms, which protect neurons from subsequently applied higher level damage. For example, exposure to mild hypoxia or mild hypoxia with ischemia can reduce hippocampal neuronal death caused by subsequent higher-level hypoxia [24, 25, 34, 36, 39, 69]. Similarly, exposure of cerebellar neurons to low concentrations of N-methyl D-aspartate (NMDA) or glutamate can reduce neuronal death caused by subsequent exposure to higher concentrations of glutamate toxins or even to other toxins like 1-methyl-4-phenylpyridinium (MPP^+) [54, 55].

The mechanisms underlying neuroprotection by pre-exposure to low-level excitotoxicity or hypoxia have been shown to require new protein synthesis [55, 69]. Low-level pre-exposure can increase mRNA or protein levels for heat shock protein 70 (HSP-70) [34] or trophic factors like basic fibroblast growth factor (bFGF) [57] and brain derived neurotrophic factor (BDNF) [56]. HSP-70, bFGF and BDNF have the capacity to reduce some forms of neuronal apoptosis [3, 29, 38, 48, 64, 65], so that increases in their synthesis after low-level pre-exposure may contribute to resistance to apoptosis. Furthermore, low-level hypoxic pre-exposure can increase levels of the anti-apoptotic protein BCL-2 [8] and decrease levels of the pro-apoptotic protein BAX [70]. In cardiac tissue, pre-exposure to low-level hypoxia with ischemia activates phosphatidylinositol 3-kinase (PI3K), which can mediate anti-apoptosis by inducing the phosphorylation of protein kinase B (Akt) [102, 109, 116]. The finding that the levels of a number of proteins that can influence apoptosis signaling are altered by low-level pre-exposure may suggest that low-level pre-exposure reduces neuronal death by modulating apoptosis signaling. It has not been determined whether low-level pre-exposure can reduce apoptosis.

In recent years, apoptosis and mitochondrial dysfunction have been increasingly implicated in neurological disease [2, 99]. Apoptosis signaling can involve increased mitochondrial membrane

permeability [31]. The increased membrane permeability is initiated in some forms of apoptosis by the opening of a multi-protein, mitochondrial membrane megapore termed the permeability transition pore complex (PTPC) [50, 89]. Sustained PTPC opening results in mitochondrial swelling, membrane rupture and the release of mitochondrial factors that signal for cell degradation [31, 89]. PTPC opening causing increased mitochondrial membrane permeability can be reflected by decreased mitochondrial membrane potential ($\Delta\Psi_M$) due a dissipation of the mitochondrial transmembrane proton gradient (see [50, 91] for reviews). BCL-2 prevents increases in mitochondrial membrane permeability, maintains $\Delta\Psi_M$ and can facilitate PTPC closure while BAX increases mitochondrial membrane permeability, decreases $\Delta\Psi_M$ and can facilitate PTPC opening (see [10, 60] for reviews).

(-)-Deprenyl and several structurally-related propargylamines reduce apoptosis in a variety of different models [12, 23, 40, 43, 52, 58, 59, 72, 73, 76, 92, 95-98, 100, 103, 107]. Similar to low-level pre-exposure, reductions in apoptosis by the propargylamines depends on new protein synthesis [98] and has been shown to involve increased synthesis of HSP70 [42, 107], trophic factors like bFGF [7, 79], NGF [84], glial cell-line derived neurotrophic factor [94] or ciliary neurotrophic factor [85] and BCL-2 [96, 107]. Deprenyl-related propargylamines prevent decreases in $\Delta\Psi_M$ in some forms of apoptosis [73, 103]. It is not known whether low-level pre-exposure can similarly affect $\Delta\Psi_M$.

It has been suggested that increased survival of myocardial cells after low-level hypoxic pre-exposure depends on signaling mechanisms involving reactive oxygen species (ROS) [16], yet it is not known whether low level pro-oxidant pre-exposure can activate protective mechanisms. We used NGF differentiated PC12 cells to show that low-level pro-oxidant exposure reduces apoptosis caused by subsequent higher-level pro-oxidant exposure or by serum and NGF withdrawal. The anti-apoptosis afforded by the low-level pro-oxidant pre-exposure requires new protein synthesis, prevents decreases in $\Delta\Psi_M$ and BCL-2 levels and is blocked by pharmacological inhibition of PI3K. Differential display PCR suggested that alterations in gene expression induced by the low-level pro-oxidant pre-exposure were very similar to those induced by deprenyl-related propargylamines suggesting that the propargylamines

may reduce apoptosis by activating similar mechanisms to those involved in mediating protection after low level pre-exposure.

Materials and Methods

Cell culture and handling. PC12 cells (American Type Culture Collection, Rockville, MD) were propagated as previously described [12, 98, 103] in minimum essential medium (MEM) with 10% horse serum and 5% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a 5% CO_2 environment. All cell culture reagents were purchased from Life Technologies (Gaithersburg, MD) unless otherwise stated. To induce neuronal differentiation, PC-12 cells were exposed to the above medium supplemented with 100 nM of 7S-NGF (Upstate Biotechnology, Lake Placed, NY).

After 5 days of exposure to serum and NGF, cultures were pretreated for 24 hours with either vehicle (HBSS) or 0.01 mM H_2O_2 in HBSS, an H_2O_2 concentration that is insufficient to cause changes in survival or morphology of the cells. PC12 cells were then washed repeatedly to remove NGF and serum borne factors and the medium was replaced with MEM without serum or NGF or with MEM containing serum and NGF as a control. Alternatively, 0.25 mM H_2O_2 was added directly to MEM containing serum and NGF. In some experiments, 10^{-9} M (-)-deprenyl, 10^{-9} M (-)-desmethyldeprenyl (DES) or 10^{-9} M CGP3466 were added concurrently with the withdrawal of serum and NGF support of the addition of 0.25 mM H_2O_2. CGP3466 is a tricyclic deprenyl analog that does not inhibit monoamine oxidase B but reduces neuronal death and apoptosis (references). For estimates of cell survival, on day 7, all cells were washed from the culture dishes, lysed and intact nuclei counted using a hemocytometer. Observers were blinded to the identity of the samples for counting.

Translational or Transcriptional Dependence. Dependence on new protein synthesis was examined as previously described [98]. Briefly, cycloheximide (10μg/ml in HBSS) or actinomycin D (3 μg/ml in HBSS) were added to cultures on both day 5 and 6 simultaneously with pretreatment (0.01 mM H_2O_2) and treatment (0.25 mM H_2O_2 or serum and NGF withdrawal) respectively ensuring that the translational or transcriptional inhibitors were present at all times during the pretreatment or treatment. Our previous studies have shown that the concentrations of cycloheximide and actinomycin D result in more than 94% inhibition of new protein synthesis.

YOYO-1 Staining to Identify Nuclei Undergoing Apoptotic Chromatin Condensation. To determine the percentage of nuclei undergoing chromatin condensation (CC), the fluorescent dye YOYO-1 (Molecular Probes, Eugene Oregon) was used as previously described [12, 13]. The cyanine family of dyes, including YOYO-1 (Molecular Probes), are symmetric dimers containing four positive charges with a very high affinity for nucleic acids. These dyes preferentially bind to C-T-A-G DNA base sequences. Prior to staining, cells grown on coverglass were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) and permeablized with -20°C methanol for 10 seconds. The cells were then incubated with YOYO-1 (1μM in 0.1 M PBS) for 30 minutes at room temperature, rinsed 3 times with PBS and mounted on glass slides with Aquamount™ (Gurr). YOYO-1 stained cells were examined using ordinary epifluorescence microscopy (excitation 488nm; emission 515nm long pass) or confocal laser scanning microscopy (excited at 488nm and detected with a 530/30 nm band pass filter) (Leica TCS 4D confocal microscope). Counts of apoptotic and normal nuclei were estimated from twenty-five 250X fields per coverslip. The x-y coordinates of each field were specified by two computer generated random numbers as described previously [103].

Phalloidin Histochemistry. PC12 cells, grown on coverglass, were fixed with 4% paraformaldehyde in 0.1M PB prior to washing with 0.1M PB, pH 7.2. The cells were then permeablized with 100% methanol at -20°C for 60 seconds and then incubated for 30 minutes at 37°C with 9μg/ml Texas Red labeled

Phalloidin in PB. Cells were then washed four times with PB and mounted onto slides with Aquamount™ (Gurr) for confocal imaging as above (excition: 568nm/ detection: 600/30nm band pass filter).

Estimation of $\Delta\Psi_M$. To estimate $\Delta\Psi_M$, PC12 cells on coverglass were incubated with 137 nM chloromethyl-tetramethylrosamine methyl ester (CMTMR; Molecular Probes) as previously described (see [13, 88, 103] for details of treatment and imaging). CMTMR binds to proteins in the mitochondrial matrix and therefore is not a Nerstian dye [51]. It concentrates in mitochondria in proportion to $\Delta\Psi_M$ but cannot leave mitochondria in response to subsequent decreases in $\Delta\Psi_M$. Furthermore, prolonged incubation of mitochondria with CMTMR can inhibit mitochondrial complex activity and may increase mitochondrial membrane permeability by acting at the PTPC [83]. Accordingly, CMTMR must be briefly incubated with live cells and the cells immediately fixed so that CMTMR mitochondrial fluorescence offers a measure of the highest level of $\Delta\Psi_M$ during the incubation period [18]. CMTMR offers the advantage that a large experimental series for a given time point can be exposed to the dye almost simultaneously and dye fluorescence measured subsequently. Furthermore, CMTMR exposed and fixed cells can subsequently be immunoreacted for specific proteins[88] or stained with nucleic acid binding dyes to determine the relationship between subcellular levels of a specific protein or the presence or absence apoptotic nuclear degradation [103].

CMTMR, in 0.1% dimethyl sulfoxide, was added directly to the wells for a period of 10 minutes followed by one wash with Dulbecco's PBS prior to fixation with 4% paraformaldehyde in 0.1M PB. Cells on cover glasses were subsequently mounted on glass slides as above. Confocal images (512 x 512 x 8 bits per pixel resolution) of CMTMR labeled PC12 cells were obtained using 568 excitation and 590 long pass detection with 32 line averaging and analyzed using Metamorph™ image analysis software as described previously [103]. Fluorescence measurements were calculated within 20 individual mitochondria per cell for 40 to 80 randomly chosen cells per condition.

Additionally, $\Delta\Psi_M$ in living partially NGF differentiated PC12 cells was estimated using tetramethylrhodamine methyl ester perchlorate (TMRM). In order to determine whether the $\Delta\Psi_M$

estimates provided by CMTMR fluorescence is reliable, we routinely examine identically treated live cells with a Nernstian dye like TMRM or JC-1 [103]. TMRM at 150nM in 0.1% dimethyl sulfoxide was incubated with living PC12 cells on 25mm coverglass in 35mm tissue culture wells for 15 minutes at 37°C. The coverglass was then removed from the tissue culture well and placed into a coverglass holder adapted to a temperature and atmosphere controlled, media circulating chamber (Medical Systems Corp. Greenvale NY USA) for imaging. The medium containing TMRM was replaced into the chamber within 90 seconds. The chamber was then placed onto the stage of an inverted fluorescence microscope coupled to a laser confocal scanning system (Leica TCS-4D) for imaging of at least 30 representative cells for each time point using a 16 count line scan. Digitally captured images were subsequently analyzed as above and as in previous studies for CMTMR labeled images[103].

Differential Display Polymerase Chain Reaction (DD-PCR). mRNA (polyA+ RNA) from PC12 cells plated at a density of 1 x 10^6 cells per 100 mm dish were purified in triplicate six hours after each of six treatments using a modification of an oligo-dT cellulose (Pharmacia LKB) chromatography procedure [21]. The treatment of PC12 cells included either HBSS vehicle (H) or 0.1mM H_2O_2 (P) on day 5 followed by vehicle, withdrawal of serum and NFG (T) (and with treatment of either 10^{-9}M (-)-deprenyl (T_D) or 10^{-9}M CGP3466 (T_C)) or 1.0mM H_2O_2 on day 6. mRNA levels were determined by UV spectrophotometry and the samples pooled for each treatment group. Reverse transcription was performed using 2 μg of mRNA and Avian Myeloblastosis Virus (AMV) reverse transcriptase in a final volume of 20 μL using degenerate 3' T_{11}VN oligonucleotide primers. A final reverse transcription concentration of 2.5 μM in a mixture consisting of 25 mM $MgCl_2$, 25 μM dNTPs in AMV reaction was used [46]. 2.0 μL of reverse transcription products were then PCR amplified in a final volume of 20 μL in the presence of the following reagents: 10 mM Tris (pH 8.3), 50 mM KCl, 9 mM $MgCl_2$, 0.5 μM 5' differential display oligonucleotide primer (50% G/C content, 10-mer length), 2.5 μM 3' degenerate oligonucleotide primer, 5 μCi {α^{35}S}-dATP and 1.5 U of *Taq* polymerase. Cycle parameters used for differential display analysis consisted of an initial 5 minute denaturation at 94°C, 30 seconds at 94° C, 2 minutes at 40° C, and 30 seconds at 72° C

for 40 cycles, followed by a final primer extension at 72° C for 5 minutes. All differential display and reverse transcription PCR reactions were performed using a Stratagene Robocycler. Reaction products from the different treatment groups were then electrophoresed through denaturing 6% polyacrylamide gels, the gels dried, and subjected to autoradiography using Kodak BioMax MR film and subsequent image analysis using Northern Eclipse (Empix Imaging, Inc, Canada).

Western Blot Analysis. PC12 cells were homogenized in lysis buffer containing 25 mM TRIS HCl, pH 8.0, 0.5% Triton X-100, 1mM EDTA, 10mM PMSF, 5mM benzamidine and 5μM leupeptin using a dounce homogenizer. The suspension was centrifuged for 5 minutes at 2000xg and the protein rich supernatant collected. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). Samples were mixed with sample buffer and heated to 95°C for 5 minutes. An aliquot of 30μL of protein was separated on a 10% SDS-PAGE gel using constant current and proteins were subsequently transferred to nitrocellulose membranes. After blocking for 1 hour in PBS containing 5% non-fat dry milk, membranes were incubated overnight in PBS containing 0.1% Tween 20, 3% non fat dry milk and the primary antibody at 4°C. Membranes were then washed with PBS and incubated with horseradish peroxidase secondary antibodies in PBS containing 0.1% Tween 20 and 3% non-fat dry milk. Immunoreactivity was detected by enhanced chemiluminescence autoradiography (Pierce, Arlington Heights, IL). The primary antibody used is a mouse monoclonal anti-Bcl-2 (1:400; Santa Cruz Biol., Santa Cruz, CA).

Results

Time Course of Cell Loss and Apoptosis with H_2O_2 treatment

Exposure of the partially NGF differentiated PC12 cells to differing concentrations of H_2O_2 revealed time courses of cell loss that varied according to H_2O_2 concentration (figure 1). As estimated by counts of intact nuclei, the highest concentration of H_2O_2, 1.0 mM, resulted in the loss of about 90% of cells by 9 hours. Half of the loss occurred in the first 3 hours after H_2O_2 addition. In contrast, exposure to

0.10 or 0.25 mM H_2O_2 induced more gradual cell loss that did not reach significance until 4.5 hours (p's <. 05) and resulted in 30 and 50% cell loss respectively by 12 hours. Exposure to 0.5 mM H_2O_2 induced a time course of cell loss that appeared to represent an average between those for 0.25 and 1.0 mM.

YOYO-1 nucleic acid staining (see figure 2) to detect nuclear CC revealed increased percentages of nuclei with evidence of apoptotic nuclear degradation for H_2O_2 concentrations of 0.25, 0.1 and 1.0 mM. Increased percentages of nuclei with apoptotic degradation were evident at the 6, 12 and 24 hour time points (p's<.05) following H_2O_2 addition. The highest percentage of nuclei with CC of 5.7% was found at 12 hours for an H_2O_2 concentration of 0.25 mM. Smaller mean values of 2.8 and 2.0 % were found for 0.1 and 1.0 mM concentrations respectively at the 12 hour time point.

Although values of 5.7% are small in comparison to the decreases in survival of about 50% for 0.25 mM H_2O_2 at the same 12-hour time point, the difference in magnitude reflects the nature of the two measures. Counts of intact nuclei offer a cumulative measure of cell loss up to the time of counting while the % of nuclei with CC offers an estimate of the percentage of cells in the final degradative stage of apoptosis at the time of counting [12]. CC proceeds through a number of phases [115] beginning with condensation or so-called margination in the peripheral portions of the nuclei near to the outer membrane. The CC then spreads to include the whole nucleus (see examples of whole nuclear involvement in figure 2A1). Condensed chromatin is then concentrated in blebs that protrude from the nucleus and break away from the main part of the nucleus as membrane wrapped apoptotic bodies. CC is evident in a number of smaller bodies that are nearby to condensation in the main nucleus and are wrapped in actin in figure 2A2. The condensed apoptotic bodies disperse and can be found as small fluorescent bodies throughout the cultures. Accordingly, nuclei with CC that can be identified with a single cell are recognizable during a limited period.

Plots like those in figure 1 for 0.25 mM H_2O_2 approximate a first-order decaying exponent. For example, an exponential fit for 0.25 mM H_2O_2 plot over the domain of 4.5 to 24 hours yields $y = 32.6 + 173.1e^{-t/5.3}$ with an r^2 value of 0.98. If we assume that the recognizable duration of nuclear CC in an

individual cell in this model is in the range of 3.5 to 4.5 hours, then exponential relationships like those for 0.25 mM H_2O_2 would predict percentages of cells with CC containing nuclei at the 12 hour time point in the range of 5 to 7 %. Accordingly, our maximum values of about 6 percent of nuclei appear to be consistent with the survival plots.

Even though figure 1 shows that the greatest and most rapid cell loss was induced by 1.0 mM H_2O_2, figure 2 shows that 1.0 mM H_2O_2 induced smaller percentages of nuclei with CC than those found for 0.1 or 0.25 mM H_2O_2. The comparatively reduced percentages in nuclei with CC may indicate that 1.0 mM H_2O_2 induced a greater proportion of necrosis and less apoptosis than the two lower concentrations.

Low Concentration H_2O_2 Pretreatment Reduces Apoptosis Induced By Subsequent Higher Concentration H_2O_2 or NGF And Serum Withdrawal

We carried out experiments in which the partially NGF differentiated PC12 cells were pre-exposed to 0.01 mM H_2O_2 on their 5th day in media with NGF and serum. As shown in figure 2, 0.01 mM H_2O_2 did not induce an increase in nuclei with CC above vehicle-treated baseline values at any time point (p's >.05). Twenty-four hours later, on day 6, the cells were exposed to 0.25 mM H_2O_2 or, alternatively, underwent trophic withdrawal by the removal of serum and NGF [12, 98, 103]. Cell loss at 24 hours with 0.25 mM H_2O_2 or serum and NGF withdrawal was significantly reduced for cells pre-exposed to 0.01 mM H_2O_2 on day 5 compared to those treated with vehicle on day 5 (figure 3A, p's < .01). Similarly, pre-exposure to 0.01 mM H_2O_2 on day 5 markedly reduced the percentage of nuclei with CC at 12 hours after the application of 0.25 mM H_2O_2 or serum and NGF withdrawal (figure 3B). The experiments established that treatment with a concentration of H_2O_2 that is insufficient to induce cell loss or apoptosis increases the cells resistance to apoptosis subsequently initiated by higher concentrations of H_2O_2 or by serum and NGF withdrawal.

(-)-Deprenyl, (-)-Desmethyldeprenyl and CGP3466 Reduce Apoptosis Similar To Pro-Oxidant Pretreatment

(-)-Deprenyl has previously been shown to reduce apoptosis in the partially NGF differentiated PC12 cells after serum and NGF withdrawal [12, 98, 103]. (-)-Desmethyldeprenyl, a major metabolite of (-)-deprenyl, also reduces apoptosis and is likely responsible for (-)-deprenyl anti-apoptosis [12, 67, 68, 96]. The tricyclic deprenyl analog, CGP3466, has been shown to reduce apoptosis in neuroblastoma cells and NGF differentiated PC12 cells [12, 40]. Similar to those findings, we found that the addition of 10^{-9} M (-)-deprenyl on day 6 reduced cell loss (figure 3A) and decreased the percentage of nuclei with CC (figure 3B) after exposure to 0.25 mM H_2O_2 or serum and NGF withdrawal. The combination of 0.01 mM H_2O_2 on day 5 and (-)-deprenyl on day 6 (figures 3A and 3B) did not significantly decrease the cell loss or the percentage of nuclei with CC relative to those found for either treatment alone (p's>.05).

Based on our finding with low-level pro-oxidant pre-exposure, it might be argued the propargylamines reduce apoptosis by mediating low level damage similar to that induced by 0.01 mM H_2O_2. Pretreatment of the cells with 10^{-9}M (-)-deprenyl, (-)-desmethyldeprenyl (DES) or CGP3466 on day 5 (figure 4) did not alter the cell loss (p's>.05) induced by 0.25 mM H_2O_2 or serum and NGF withdrawal on day 6, which argues against the possibility that the propargylamines reduce apoptosis by inducing low level damage.

Low-level Pro-oxidant Damage Protects PC12 cells Against Apoptosis By A Mechanism Requiring New Protein Synthesis.

Experiments using actinomycin D (3 μg/ml) and cycloheximide (10 μg/ml) to inhibit either transcription or translation respectively showed that the protective effect of low level H_2O_2 pre-exposure required new protein synthesis (figure 4). We have previously shown that actinomycin D and cycloheximide concentrations of 3 μg/ml and 10 μg/ml respectively decreased new protein synthesis in the partially NGF differentiated PC12 cells by more than 90% and were optimal for reducing new protein synthesis in the partially NGF differentiated PC12 cells [98]. Increases in survival induced by day 5 pre-

exposure to 0.01 mM H_2O_2 were completely eliminated by the addition of actinomycin D or cycloheximide on both day 5 and day 6. In contrast, actinomycin D or cycloheximide treatment did not alter the survival of cells after vehicle treatment on day 5 followed by 0.25 mM H_2O_2 on day 6. These finding are consistent with previous evidence that protection by low-level pre-exposure to hypoxia or excitotoxicity requires new protein synthesis [55, 69].

Low Level Pro-Oxidant Pre-Treatment Prevents The Reduction In $\Delta\Psi_M$ Induced By Trophic Withdrawal

The effect of low-level H_2O_2 pre-exposure on $\Delta\Psi_M$ was investigated using CMTMR with laser scanning confocal microscopy (LCSM) (see [13, 28, 32, 88, 103] for details examples of our previous use of CMTMR with LSCM to estimate $\Delta\Psi_M$). In an initial series of experiments, we treated the cells with varying concentrations of carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), which collapses the proton gradient across the mitochondrial inner membrane, resulting in a concentration dependent dissipation of $\Delta\Psi_M$ in PC12 cells [19] and other neuron-like cells [22]. FCCP offers a method of calibrating decreases in $\Delta\Psi_M$ between experiments and for different treatments [74]. We utilized FCCP concentrations of 0.5, 5.0 and 10.0 mM and measured $\Delta\Psi_M$ at 3 hours after FCCP addition using two different potentiometric dyes, CMTMR and tetramethylrhodamine methylester (TMRM). Living cells on coverslips in a chamber placed on an inverted microscope on laser confocal system were incubated with TMRM at 2 hours and forty-five minutes to 3 hours and 15 minutes after FCCP addition (see [103] for details of the system). Images were taken at random coordinates on the coverslips over the 30 minute imaging period for each coverslip. Alternatively, cells on coverslips were treated with CMTMR for 10 minutes at 2 hours and 55 minutes after FCCP addition and then fixed immediately.

Both dyes revealed broad distributions of mitochondrial fluorescence (see figure 5) similar to those we have previously shown with CMTMR and JC-1 [13, 88, 103]. The broad distributions reflected variations in fluorescence for mitochondria within individual cells and variations in fluorescence across the cell populations. With increasing concentrations of FCCP, the distributions progressively shifted to

lower values and became narrower as shown by their means and standard deviations. When the mean values and SEMs for each distribution were normalized against the mean values for the vehicle treated cells for the same dye (plot on right hand side of figure 5), the percentage decreases in CMTMR and TMRM fluorescence were similar for increasing FCCP concentrations.

Figure 6 provides typical examples of CMTMR fluorescence on LSCM micrographs together with the corresponding CMTMR fluorescence distributions. Cells that were treated with vehicle on day 5 followed by serum and NGF withdrawal on day 6 showed dramatically decreased $\Delta\Psi_M$ at 6 hours after the onset of withdrawal (compare micrographs and distributions labeled H5-T6 to that labeled H5-H6). As previously shown [103], addition of 10^{-9} M (-)-deprenyl at the initiation of trophic withdrawal on day 6, prevented a large proportion of the decrease in $\Delta\Psi_M$ (compare micrographs and distributions labeled H5-T6 to H5-$T6_D$, $p<.05$). Pretreatment with 0.01 mM H_2O_2 on day 5 did not induce changes in $\Delta\Psi_M$ in cells treated with vehicle on day 6 (compare micrographs and distributions labeled P5-H6 to H5-H6, $p>.05$). In contrast, similar to (-)-deprenyl treatment on day 6, pre-exposure to 0.01 mM H_2O_2 on day 5 prevented much of the decrease in $\Delta\Psi_M$ induced by serum and NGF withdrawal on day 6 (compare micrographs and distributions labeled P5-T6 to H5-T6, $p< .001$). Pretreatment with 0.01 mM H_2O_2 on day 5 followed by serum and NGF withdrawal and addition of 10^{-9} M (-)-deprenyl on day 6 resulted in $\Delta\Psi_M$ levels that were similar to those for (-)-deprenyl treatment on day 6 alone (compare micrographs and distributions labeled P5- $T6_D$ to H5-$T6_D$, $p>.05$).

These results show that pre-exposure to 0.01 mM H_2O_2 on day 5 prevents most of the reduction in $\Delta\Psi_M$ that occurs in the partially NGF differentiated PC12 cells after serum and NGF withdrawal and which precedes the appearance of apoptotic nuclear degradation [103]. The loss of $\Delta\Psi_M$ in serum and NGF withdrawn partially differentiated PC12 cells is accompanied by an increase in intramitochondrial Ca^{2+} [103] and may reflect increased mitochondrial membrane permeability [91]. Low level pro-oxidant damage may therefore activate a program of new protein synthesis that includes proteins that act to maintain $\Delta\Psi_M$, possibly by maintaining mitochondrial membrane impermeability.

Evidence That Low Level Pro-oxidant Damage And Treatment with Deprenyl Analogs Activate A Similar Program of New Protein Synthesis

We used DD-PCR as a means of comparing the overall patterns of gene expression between different experiment groups. We examined the mRNAs for tissue taken from the cultures at 6 hours on day 6. Figure 7 presents typical findings for three different primers. In each panel, the bands in lane 1 are for cells treated with vehicle on day 5 and again on day 6. Lane 3 and 4 in each panel are for cells treated with vehicle on day 5 followed by serum and NGF withdrawal or exposure to 0.25 mM H_2O_2 respectively on day 6. Lane 2 represents cells exposed to 0.01 mM H_2O_2 on day 5 and trophic withdrawal on day 6 while lanes 5 and 6 represent cells treated with vehicle on day 5 followed by both serum and NGF withdrawal and (-)-deprenyl or CGP3466 treatment respectively on day 6.

The asterisks at the right side of each panel mark bands that are common to lanes 1, 2, 5 and 6 or lanes 2, 5 and 6 but were not present in lanes 3 or 4. That is, they include PCR products found in day 5 and day 6 vehicle treated cells (lane 1), serum and NGF withdrawn cells on day 6 exposed to low level pro-oxidant damage on day 5 (lane 2) or serum and NGF withdrawn cells treated with (-)-deprenyl or CGP3466 on day 6 (lanes 5 and 6 respectively). Importantly, the asterisk marked bands were not present for cells that were serum and NGF withdrawn without pro-oxidant pretreatment or treatment with a deprenyl–related propargylamine (lanes 3 and 4).

BCL-2 Is Increased By Low Level Pro-oxidant Pre-exposure. Previous studies have shown that deprenyl-related propargylamines can induce increased synthesis of a number of proteins including BCL-2 [96, 107]. BCL-2 overexpression can reduce apoptosis, and prevent the decreases in $\Delta\Psi_M$ that are associated with some forms of apoptosis (see [60, 91] for reviews). We examined western blots for BCL-2 using lysates from NGF differentiated PC12 cells harvested at 6 hours after day 6 treatments for BCL-2 immunodensity (figure 8). Cells that were treated with vehicle on day 5 and serum and NGF withdrawn on day 6 showed a marked decrease in BCL-2 immunodensity that was prevented by exposure to 0.01 mM H_2O_2 on day 5.

Addition of propargylamines at 10^{-9} M on day 6 did not detectably alter the increase in BCL-2 immunodensity provided by exposure to 0.01 mM H_2O_2 on day 5.

Inhibition Of PI3K Prevents Protection By Low Level Pro-oxidant Pre-exposure. The PI3K/Akt pathway has been shown to oppose a number of forms of apoptosis in PC12 cells [1, 4, 80, 81, 86, 104, 105], including apoptosis induced by hypoxia and NGF withdrawal. Wortmannin and LY294002 inhibit PI3K and therefore decrease phosphorylation of Akt. We treated the PC12 cells with 75 μM of LY294002, which has been shown to completely inhibit PI3K in PC12 cells [44, 86, 87, 110], on both day 5 and day 6. We found that pharmacological inhibition of PI3K prevented the increased survival induced by pre-exposure to 0.01 mM H_2O_2 .

Discussion

The phenomenon of cellular protection by pre-exposure to sub-lethal levels of damaging agents or events prior to a subsequent applied severe insult was first described for ischemia pre-treated cardiac tissue [66]. Subsequently, pre-exposure has been described in a number of other systems in various non-neuronal and neuronal cell types [11, 24, 27, 63, 106, 108]. Various pre-exposure insults have been utilized including TNFα treatment [26], mitochondrial respiratory chain inhibition [106] and $FeSO_4$ to generate hydroxyl radicals and xanthine/xanthinoxidase to generate superoxide radicals [77]. Elements proposed to contribute to the protection have included reactive oxygen species [77], ceramide [26, 47], p53 [101], increased Bcl-2 gene expression [70], BAX [62], nitric oxide [25] and mitochondrial K_{ATP} channel activation [93]. Previous studies have implicated a number of proteins in nervous system protection including NFκB [61], HSP-70 [34], bFGF [57], BDNF [56], BCL-2 [8], BAX [70], p53 and p21 [101]. Others have suggested the involvement of lipid factors like ceramide [26, 47].

In the present study, we have shown that pro-oxidant pre-exposure increases the survival of neuronal-like cells and that the increase in survival is due to a reduction in apoptosis. The decrease in apoptosis induced by low-level pro-oxidant pre-exposure was accompanied by a reduction in the $\Delta\Psi_M$

dissipation that is an early feature of many forms of apoptosis [91] and required the synthesis of new proteins. Apoptosis in serum and NGF withdrawn or H_2O_2 treated PC12 cells has been shown to involve decreases in BCL-2 protein levels and can be prevented or reduced by increased bcl-2 expression [35, 53, 82]. We found that low-level pro-oxidant pre-exposure prevented BCL-2 protein level decrease associated with serum and NGF withdrawal.

The PTPC has been shown to contribute to increased mitochondrial membrane permeability in some forms of apoptosis. The PTPC has been defined as a voltage-dependent, cyclosporin A sensitive, high conductance mitochondrial membrane channel [5]. Sustained PTPC opening affects mitochondrial respiration due to loss of pyridine nucleotide-linked substrates from the mitochondrial matrix and can induce osmotic swelling of mitochondria with fracture of their inner and outer membranes [6, 31]. Members of the BCL-2 family of proteins appear to mediate part of their pro- or anti-apoptotic actions by localizing to mitochondrial membranes, in part in association with the PTPC [10, 60, 111]. The association of BCL-2 or BCL-X_L with the PTPC seems to facilitate PTPC closure and results in the maintenance of $\Delta\Psi_M$ [10, 41, 112, 113]. The maintenance of $\Delta\Psi_M$ that we have shown after pro-oxidant pre-exposure therefore likely indicates the maintenance of impermeability of the inner mitochondrial membrane but does not necessarily indicate that outer mitochondrial membrane has been maintained [15].

The release of factors that induce apoptotic nuclear degradation requires outer membrane permeabilization [15, 31]. At least two factors that signal for apoptotic degradation are released from the intermembranous space across the outer mitochondrial membrane, cytochrome C and apoptosis initiation factor (AIF), a 50 kD flavoprotein. Cytochrome C interacts with Apaf-1 [9, 45, 119], dATP/ATP and pro-caspase 9 to form a complex known as the apoptosome, in which pro-caspase 9 is converted to caspase 9. Caspase 9 then converts pro-caspase 3 to active caspase 3. Activated caspase 3 in turn activates DNA fragmentation factor [49], an endonuclease activator that enables DNA cleavage and also cleaves other proteins such as lamin, and fodrin [119]. AIF induces DNA loss, peripheral CC and digestion of chromatin into 50 kbp-fragments [90].

Several studies of mitochondria *in situ* have shown that PTPC opening and decreased $\Delta\Psi_M$ can be associated with the induction of apoptotic degradation in some apoptosis models [71, 78]. Examination of individual mitochondria *in situ* using LCSM has shown that decreased $\Delta\Psi_M$ can accompany cytochrome C release into the cytosol [30]. Mitochondrial BCL-2 appears to block the release of the mitochondrial factors that signal for apoptotic degradation, possibly due to its capacity to facilitate PTPC closure and maintain $\Delta\Psi_M$ (see [14] for a recent review of the role of the PTPC in cell death). Accordingly, the maintenance of BCL-2 that we have shown after pro-oxidant pre-exposure may result in maintained outer mitochondrial membrane impermeability, which may contribute to reduced apoptosis by curtailing apoptotic factor release from mitochondria.

Deprenyl-related propargylamines had previously been suggested to reduce apoptosis through mechanisms involving a similar maintenance of BCL-2 levels [96, 107] and $\Delta\Psi_M$ [73, 103, 114] to those we have shown for pro-oxidant pre-exposure. Our finding that the changes in gene expression induced by low-level pro-oxidant pre-exposure and the propargylamines is consistent with the possibility of common anti-apoptotic mechanisms. The propargylamines have been shown to maintain $\Delta\Psi_M$ and BCL-2 levels similarly to the pro-oxidant pre-exposure.

In this study, we have shown that a pharmacological inhibitor of PI3K blocks the capacity of pro-oxidant pre-exposure to reduce apoptosis in the partially differentiated PC12 cells. We have also found that inhibitors of PI3K block the capacity of the propargylamines to reduce apoptosis (Chalmers-Redman and Tatton, unpublished observations). Accordingly, both the propargylamines and low-level pro-oxidant pre-exposure require PI3K activity for anti-apoptosis.

Many growth factors, including NGF, affect cellular survival through receptor dependent activation of PI3K [20]. Akt phosphorylation by growth factor activation of PI3K reduces apoptosis by decreasing cytochrome C release from mitochondria [37]. Part of the capacity of phosphorylated Akt to maintain mitochondrial membrane impermeability is likely mediated through BCL-2 or BCL-XL. Phosphorylated Akt upregulates bcl-2 expression through the cAMP-response element-binding protein [75] and can also upregulate bcl-2 expression through pathways that involve NF-κB [33, 118]. It also can increase

BCL-2 interactions with mitochondrial membranes by phosphorylating BAD and thereby preventing the heterodimerization of BAD with either BCL-2 or BCL-XL [17, 117].

The actions of phosphorylated Akt on BCL-2 levels may not fully explain the capacity of low-level pro-oxidant pre-exposure to maintain $\Delta\Psi_M$ and reduce apoptosis in our experiments. Recently, Akt and BCL-XL have been shown to mediate distinct effects on mitochondria in growth factor withdrawn cells [74]. Phosphorylated Akt has been shown to increase glucose transporter and glycolytic activity in concert with the maintenance of $\Delta\Psi_M$, while BCL-XL maintains $\Delta\Psi_M$ independently of glycolytic maintenance. As well as the glycolytic actions of Akt, it has been reported to reduce the levels of superoxide radicals in NGF differentiated PC12 cells treated with the mitochondrial complex I inhibitor, MPP^+ [81] and to inhibit the activation of caspase 9 and caspase 3 by cytochrome c [116]. Accordingly, pro-oxidant pre-exposure may reduce apoptosis through the activation of a number of signaling pathways that act at both pre- and post-mitochondrial levels.

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Figure Legend

Figure 1. *Time Course of H_2O_2 induced cell death.* Time courses for the death of neuronally differentiated PC12 cells following exposure to varying concentrations of H_2O_2 on day 6. PC12 cells were treated with vehicle (squares), 0.1mM H_2O_2 (circles), 0.25 mM H_2O_2 (triangles), 0.5 mM H_2O_2 (inverted triangles) and 1.0 mM H_2O_2 (diamonds). Counts of intact nuclei were made at the time of H_2O_2 addition (0 hours) and every 90 minutes thereafter until 18 hours. Points represent the mean of 3 samples each from 4 separate experiments ± SEM.

Figure 2. *Apoptotic cell death due to H_2O_2 exposure.* YOYO-1 staining of apoptotic nuclei. Row A presents laser confocal micrographs of YOYO-1 stained nuclei at various stages of the progression of apoptotic nuclear degradation. Row B shows the corresponding actin staining for identical image field to those in row A (A1 corresponds to B1 and A2 corresponds to B2). Panel A1 shows 2 cells undergoing apoptosis (labeled a), 1 cell undergoing mitosis (labeled m) and 3 normal cells (labeled n). Panel A2 shows later stages of nuclear apoptotic degradation than that shown in A1. Note the formation of two apoptotic bodies (labeled ab) near to the condensed nuclear remnant and the actin surrounding the nuclei and apoptotic bodies in corresponding cells in B1, and B2. The graph in panel C shows the percentage of cells with YOYO-1 stained nuclei exhibiting CC from 25 randomly-chosen 100x fields per coverslip for 4 coverslips at each time point (3, 6, 12 and 24 hours) after exposure to various concentrations of H_2O_2. Plotted values are means +/- SEMs.

Figure 3. *Low-level H_2O_2 pre-exposure reduces cell loss following higher lever pro-oxidant exposure.* Estimation of cell survival by counts of intact nuclei at 24 hours (A.) and nuclei with apoptotic degradation at 12 hours (B.) after pro-oxidant exposure or serum and NGF withdrawal, which was preceded by vehicle or low level pro-oxidant exposure 24 hours earlier. On the abscissa's, the top row indicates day 5 pre-treatments and bottom row indicates day 6 treatments. Pretreatments on day 5 include

HBSS (H) as the vehicle control or H_2O_2 (P) at 0.01mM. Treatments on day 6 include HBSS (H) as vehicle control, H_2O_2 (P) at 0.25mM, serum and NGF withdrawal (T) and (-)-deprenyl addition (subscript D) at 10^{-9} M. The ordinate in A. indicates the number of intact nuclei at 24 hours following the day 6 treatments while that in B. shows the percentage of nuclei with CC at 12 hours after day 6 treatments. Bars represent percent of control and error bars represent standard errors of the mean.

Figure 4. *The reduction in cell loss by low-level H_2O_2 requires the synthesis of new proteins.* Estimation of cell survival following H_2O_2 (P) low level exposure (0.01 mM) on day 5 followed by H_2O_2 (0.25 mM) with or without cycloheximide or actinomycin D addition on days 5 and 6 to determine the dependence of the increased survival on new protein synthesis. As well, the effects of pre-treatment with known anti-apoptotic agents, (-)-desmethyldeprenyl (DES) at10^{-9} M, CGP3466 at 10^{-9} M and (-)-deprenyl at 10^{-9}M are presented. As in figure 3, the top row of the abscissa legend indicates the pre-treatment on day 5 and bottom row indicates the day 6 treatment. Treatments and pretreatments include HBSS (H), H_2O_2 (P), cycloheximide (X), actinomycin D (A), (-)-deprenyl (D), (-)-desmethyldeprenyl (S), and CGP3466 (C). Bars represent mean percent of intact PC12 cell nuclei relative to control. Error bars represent SEMs.

Figure 5. *Disruption of DYM with FCCP results in a graded decrease in CMTMR fluorescence distribution.* Part A. presents changes distributions of mitochondrial fluorescence for CMTMR (left hand column of distributions) and TMRM (right hand column of distributions) measured using LCSM for increasing concentrations of the protonophore, FCCP. The values above each distribution indicate the FCCP concentration and the mean and standard deviation values for the distribution. The number measurements used to construct each distribution varied between 441 and 687. Part B. presents the mean +/- SEM values for the two columns of distributions normalized against the mean values for the vehicle treated cells examined by the two dyes and expressed as percentages. FCCP concentrations are plotted using $\log_{10}$ base values.

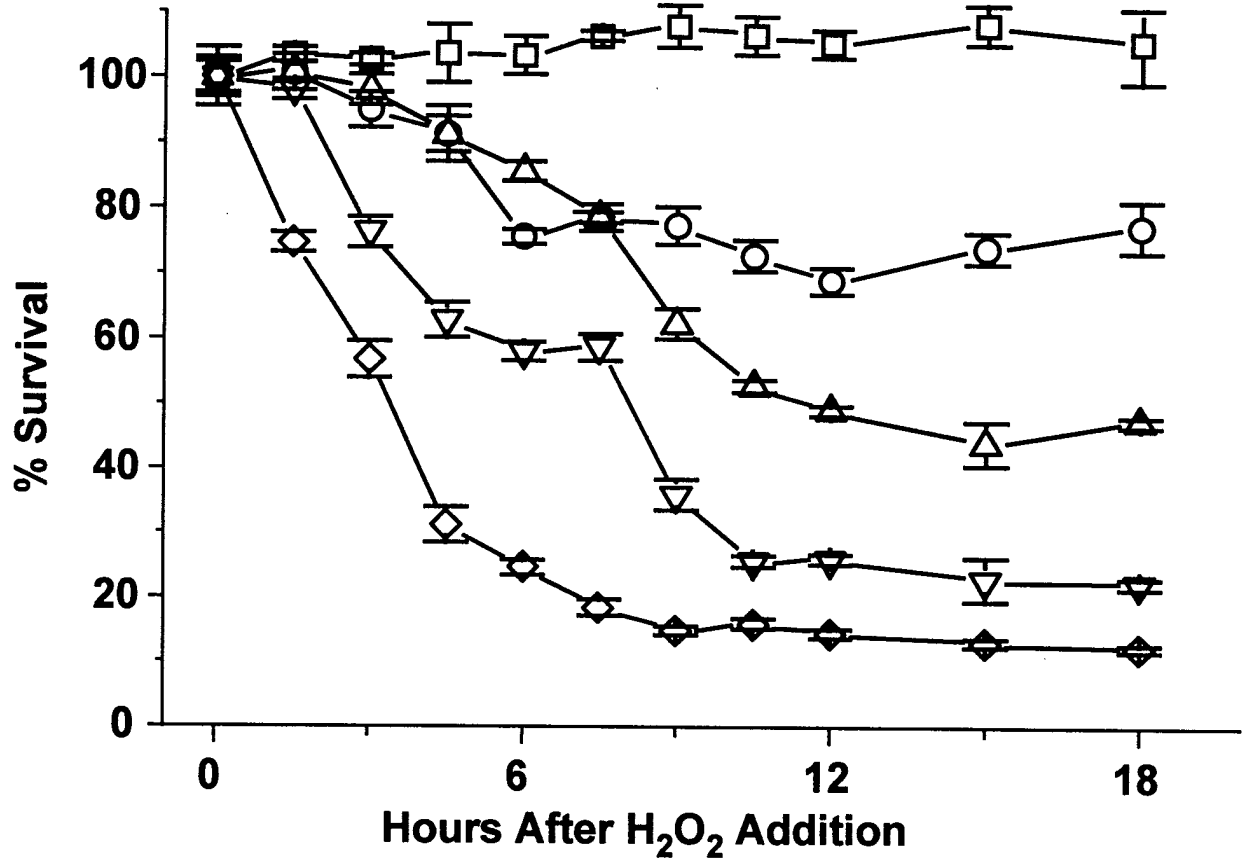
Figure 6. *Low-level H_2O_2 induced reductions in cell loss involves the maintenance of $\Delta\Psi_M$.* Part A-F present typical LCSM images of CMTMR fluorescence of partially NGF differentiated PC12 cells exposed to different treatments and pre-treatments. A represents vehicle pretreatment on day 5 followed by vehicle treatment on day 6. B represents vehicle pretreatment and trophic withdrawal on day 6. Trophically withdrawn cells exposed to (-)-deprenyl on day 6 after vehicle pretreatment on day 5 are shown in panel C. Panels D-F were pretreated on day 5 with 0.01 mM H_2O_2 followed by no treatment, trophic withdrawal and trophic withdrawal with (-)-deprenyl respectively. The intensity of the staining provides an indication of the $\Delta\Psi_M$. The distributions in part G. below the LCSM micrographs are organized so as to correspond in position to the micrographs and are identically labeled to indicate day 5 and day 6 treatment. The bars in part H. present the mean+/-SEMs for each of the distributions in part G.

Figure 7. *Low-level H_2O_2 pre-exposure includes similar gene changes to that observed following propargylamine exposure.* Three different primers were used with differential display (DD) PCR to compare gene products for the differently treated PC12 cells. Lane 1 represents untreated partially NGF differentiated PC12 cells harvested on day 6. Lane 2 represents the PC12 cells pre-treated on day 5 with 0.1 mM H_2O_2 followed by withdrawal of serum and NGF on day 6. Lanes 3, 5 and 6 represent vehicle pretreatment on day 5 and withdrawal of serum and NGF on day 6. Serum and NGF withdrawn cells represented on lanes 5 and 6 were treated with (-)-deprenyl and CGP3466 respectively. Cells used for lane 4 were treated with vehicle on day 5 and 0.25 mM H_2O_2 on day 6. This and other DD PCR showed a high correspondence of bands for cells pre-exposed to low level H_2O_2 on day 5 and those treated on day 6 with 10^{-9} M (-)-deprenyl or CGP3466 (abbreviations are the same as those for figures 3 and 4). Corresponding bands for pro-oxidant pre-exposure (lane 2) and propargylamine treatment (lanes 5 and 6) are marked by asterisk.

Figure 8. *Low-level H_2O_2 on day 5 results in maintenance of BCL-2 protein levels on day 6.*Western blot for BCL-2 immunodensity at 6 hours after treatment on day 6. The blot shows a marked decrease in BCL-

2 immunodensity for cells that were treated with vehicle on day 5 and were serum and NGF withdrawn on day 6 (labeled H-T). In contrast, low level pro-oxidant pre-exposure on day 5 followed by serum and NGF withdrawal on day 6 did not show a detectable decrease in BCL-2 immunodensity (labeled P-T). Abbreviations are the same as those used in figures 3 and 4.

Figure 9. *Inhibition of PI3K prevents the protection afforded by low-level H_2O_2 exposure.* Estimation of cell survival by counts of intact nuclei at 24 hours after pro-oxidant exposure or serum and NGF withdrawal, which was preceded by vehicle or low level pro-oxidant exposure 24 hours earlier. On the abscissa's, the top row indicates day 5 pre-treatments and bottom row indicates day 6 treatments. Pretreatments on day 5 include HBSS (H) as the vehicle control, H_2O_2 (P) at 0.01mM or H_2O_2 supplemented with LY294002 (75μM). Treatments on day 6 include HBSS (H) as vehicle control, H_2O_2 (P) at 0.25mM, serum and NGF withdrawal (T) and supplementation of P or T with LY294002 (P_L and T_L respectively). Bars represent percent of control and error bars represent standard errors of the mean.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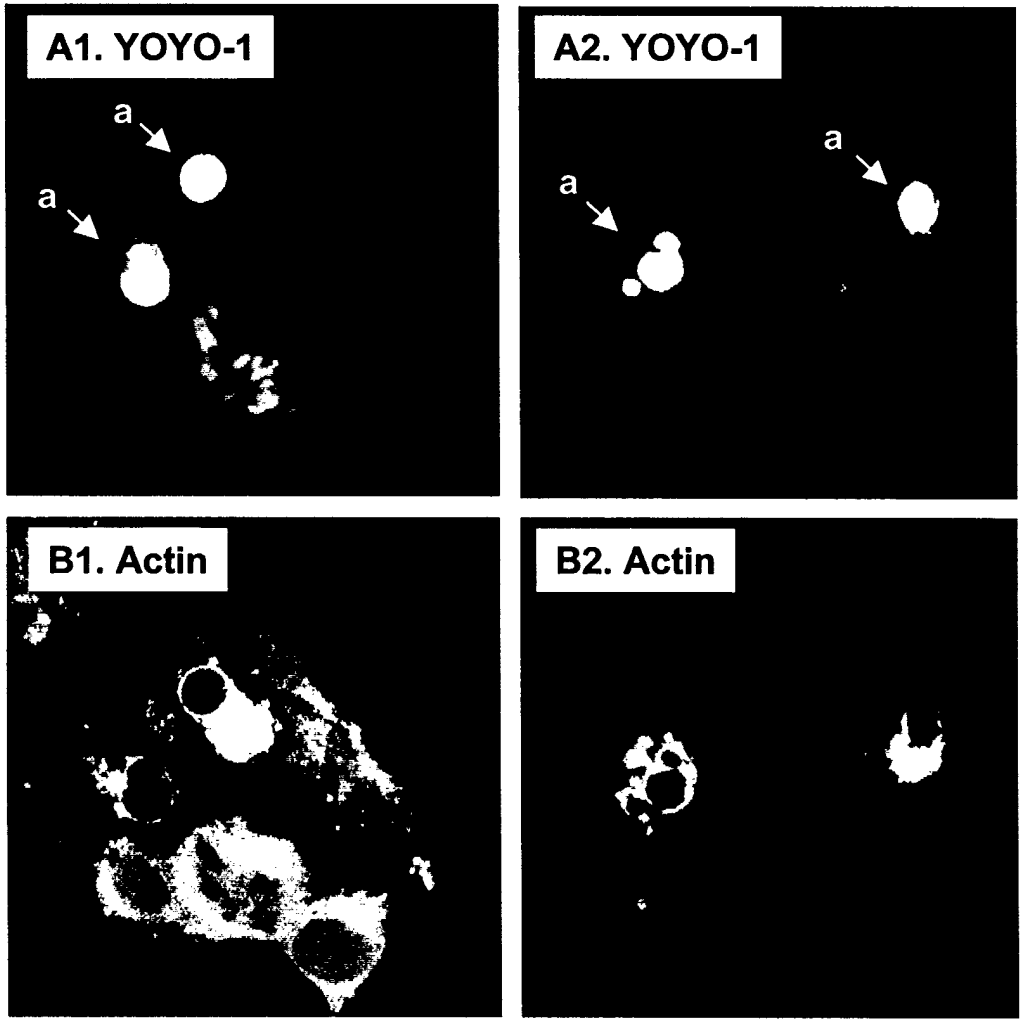
0.25 mM H_2O_2 Added
0.5 mM H_2O_2 Added
1.0 mM H_2O_2 Added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a
a
A2. YOYO-1
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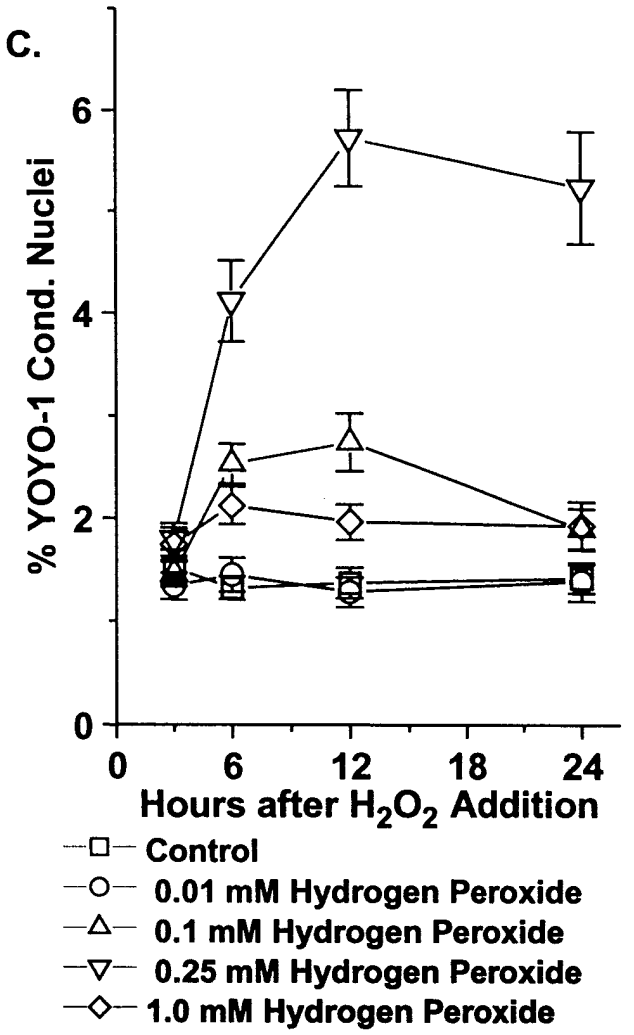
a
B1. Actin
B2. Actin


C.
% YOYO-1 Cond. Nuclei
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6
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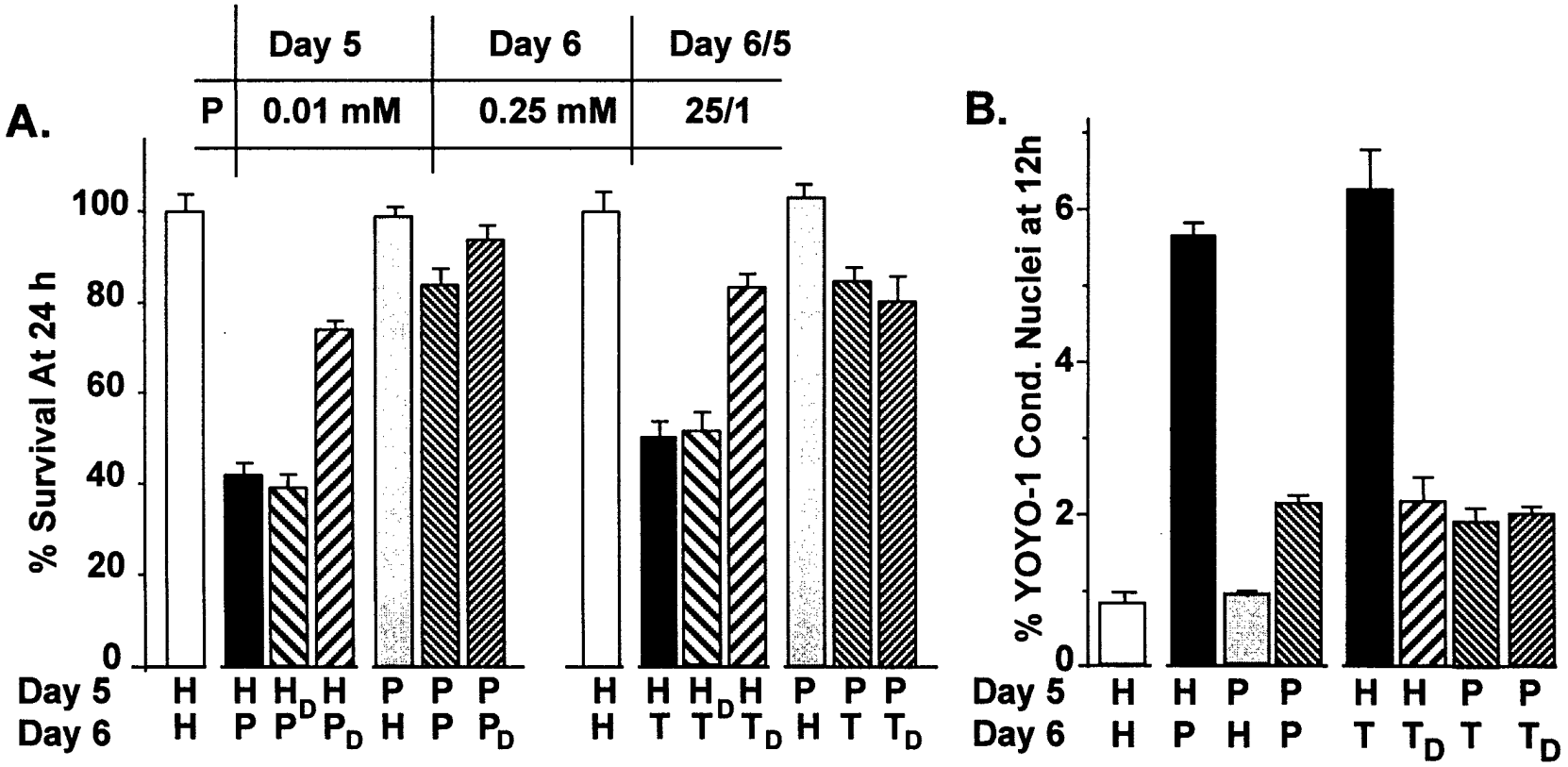
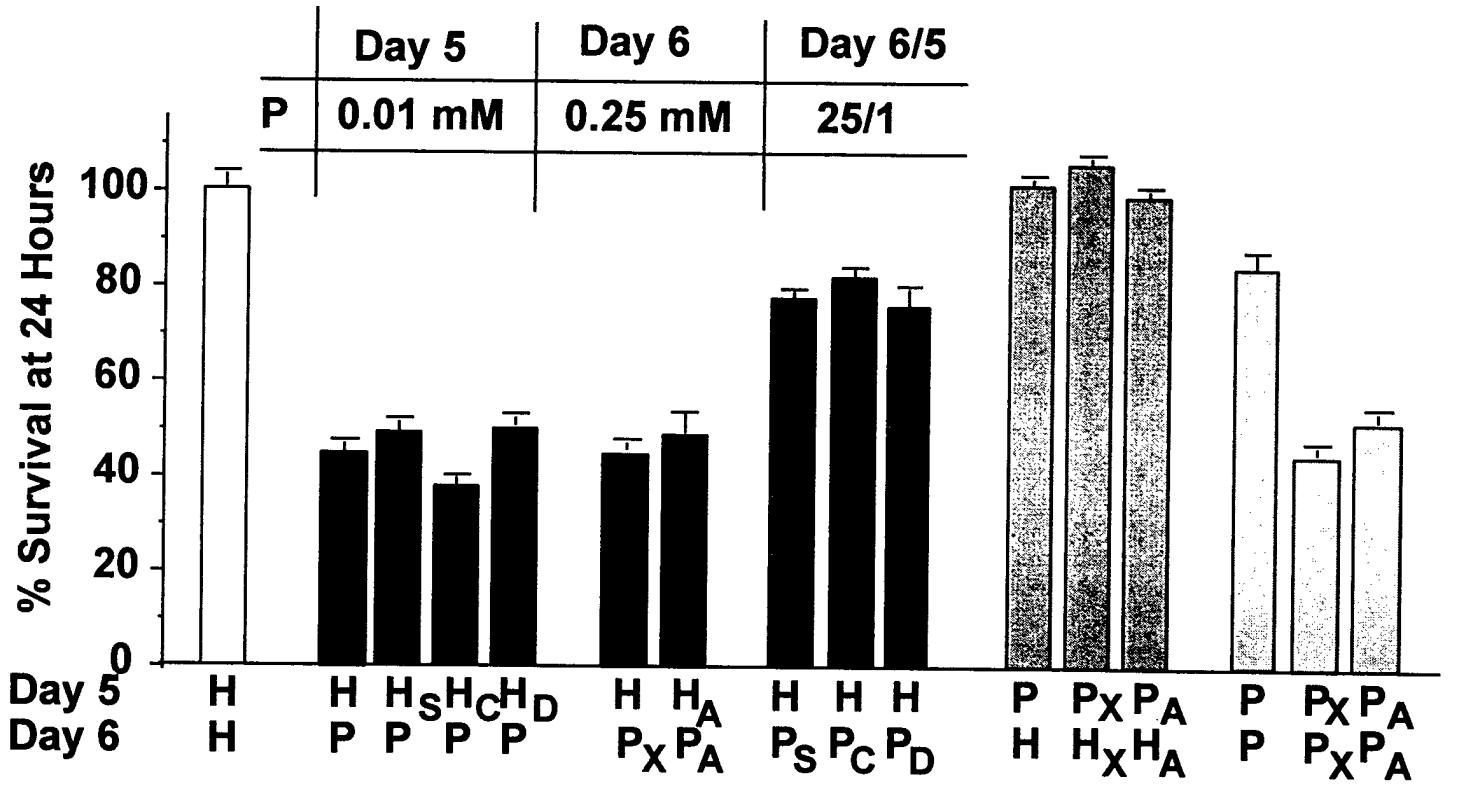
Hours after H_2O_2 Addition
Control
0.01 mM Hydrogen Peroxide
0.1 mM Hydrogen Peroxide
0.25 mM Hydrogen Peroxide
1.0 mM Hydrogen Peroxide



H - HBSS P - Hydrogen Peroxide T - NGF and Serum Withdrawal

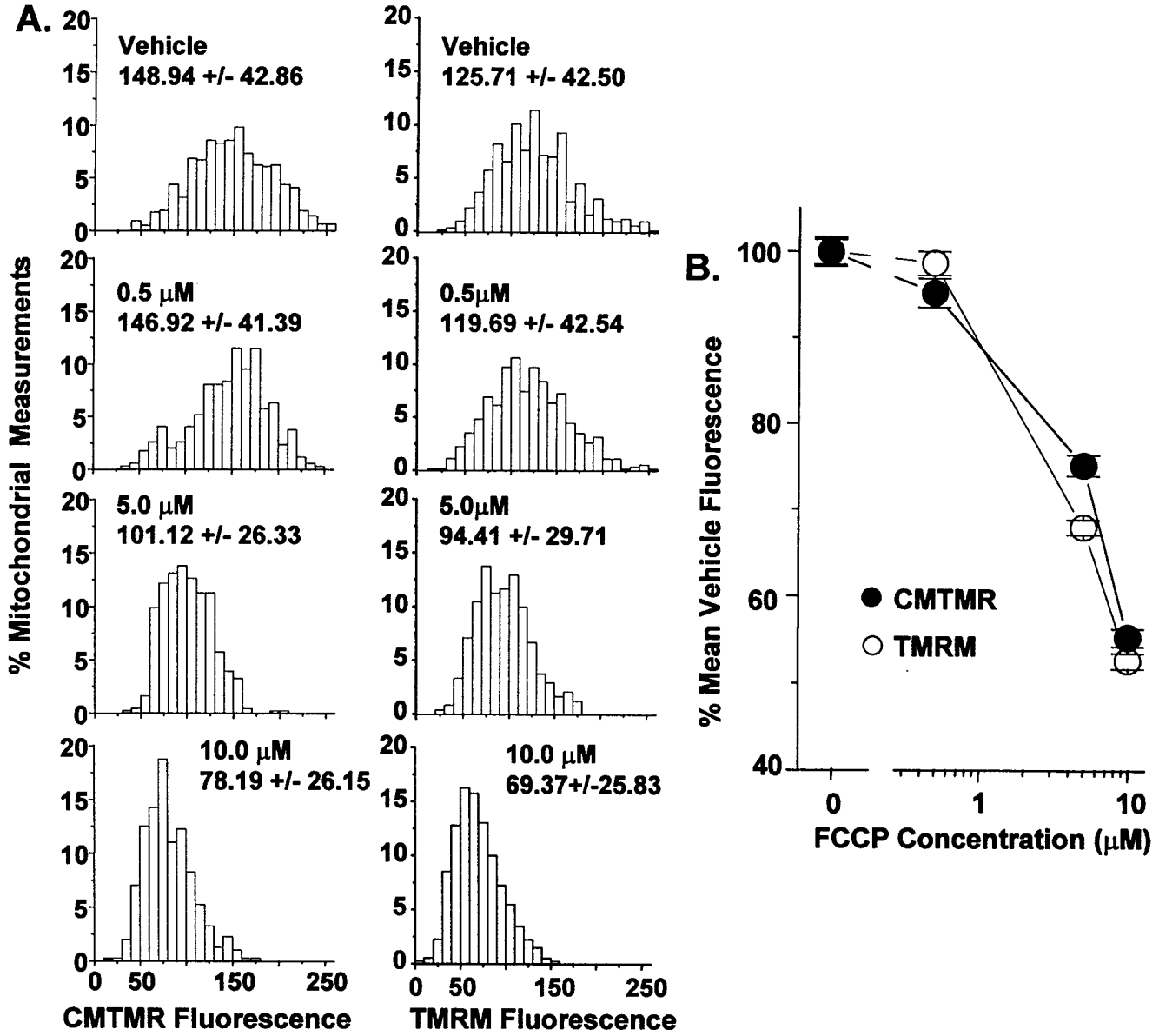
T_D or P_D - NGF and Serum Withdrawal or Hydrogen Peroxide plus (-)-Deprenyl (10^{-9} M)

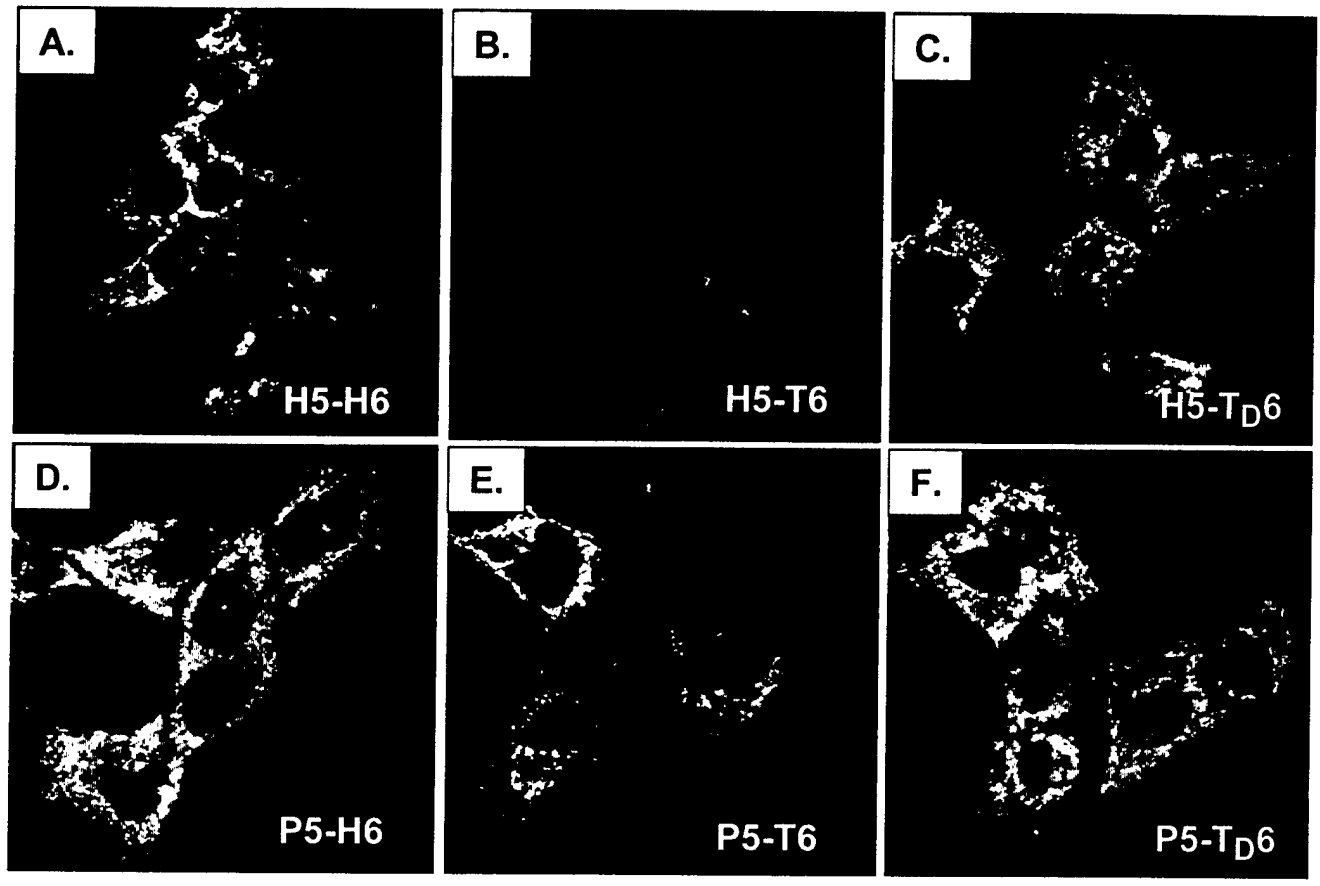


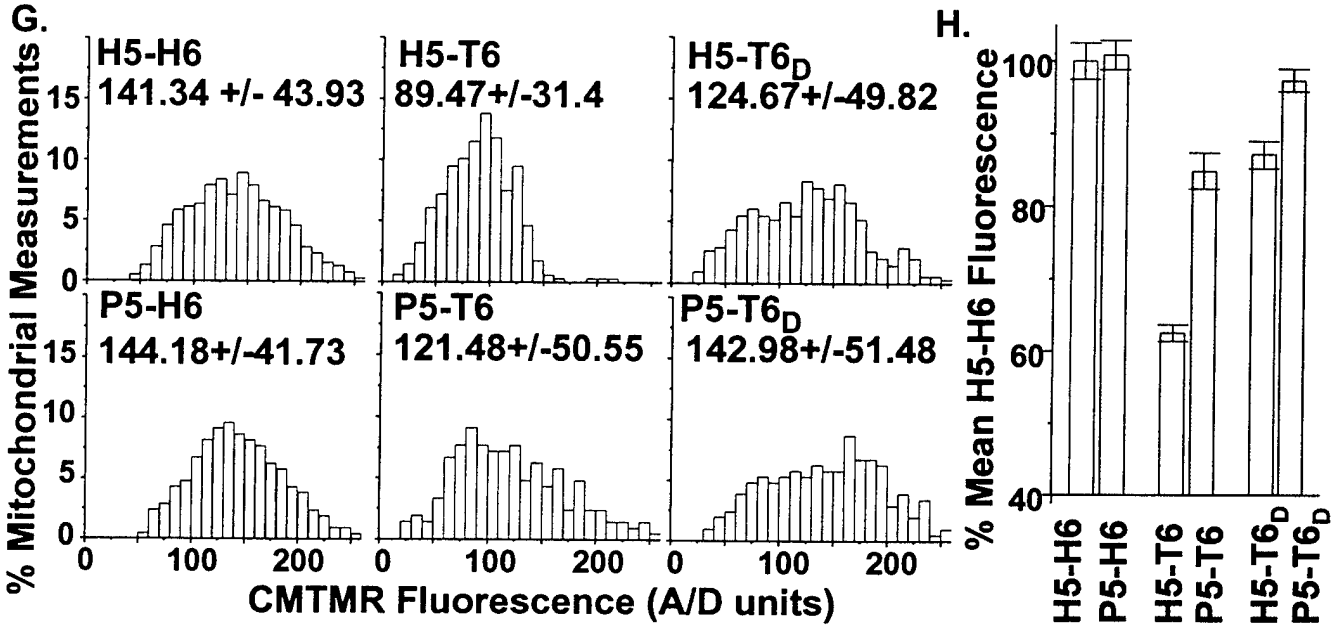
H - HBSS P - Hydrogen Peroxide X - Cycloheximide (10 μg/ml)

A - Actinomycin (3 μg/ml) S - DES (10^{-9} M) C - CGP3466 (10^{-9} M)

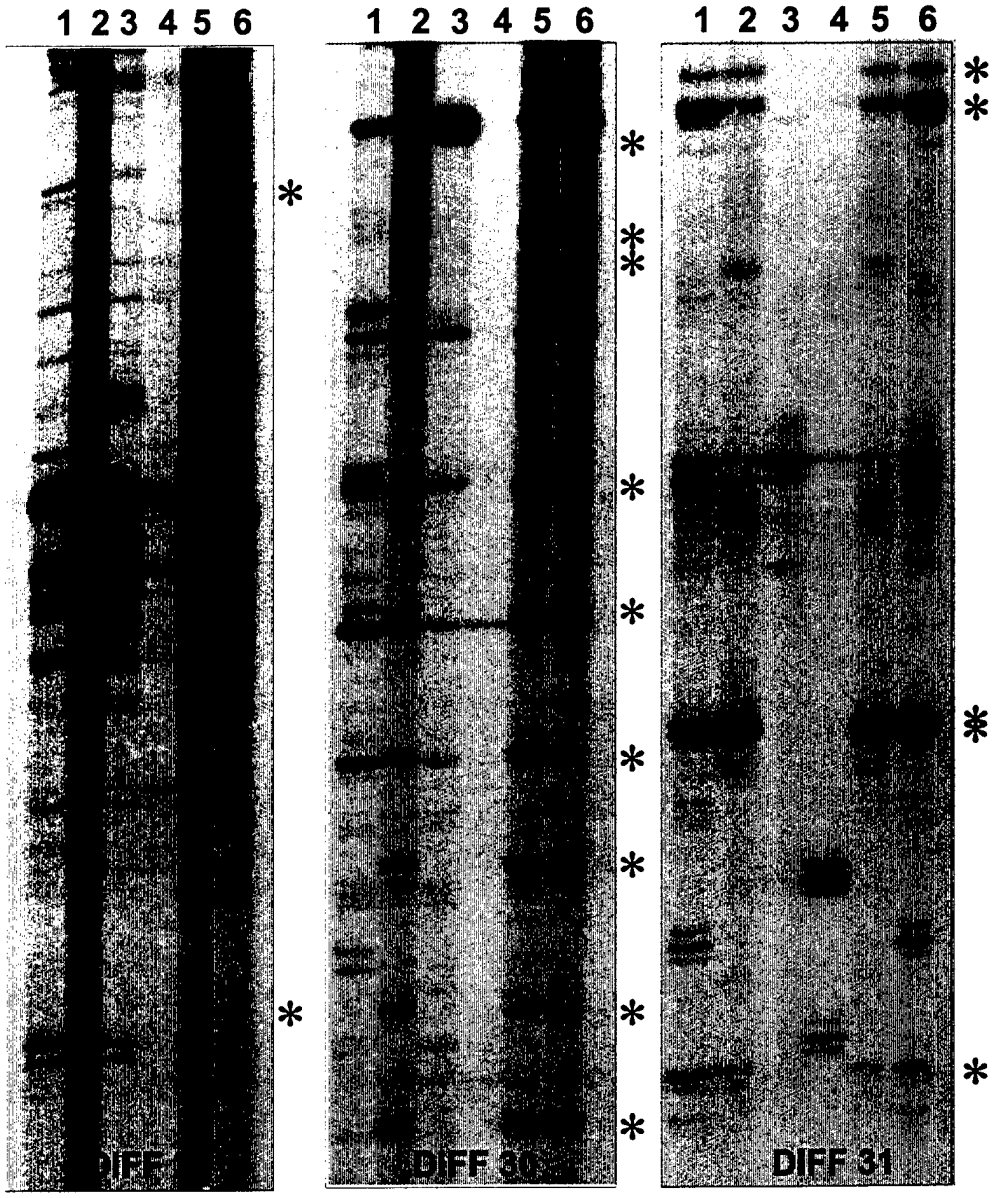
D - Deprenyl (10^{-9} M)

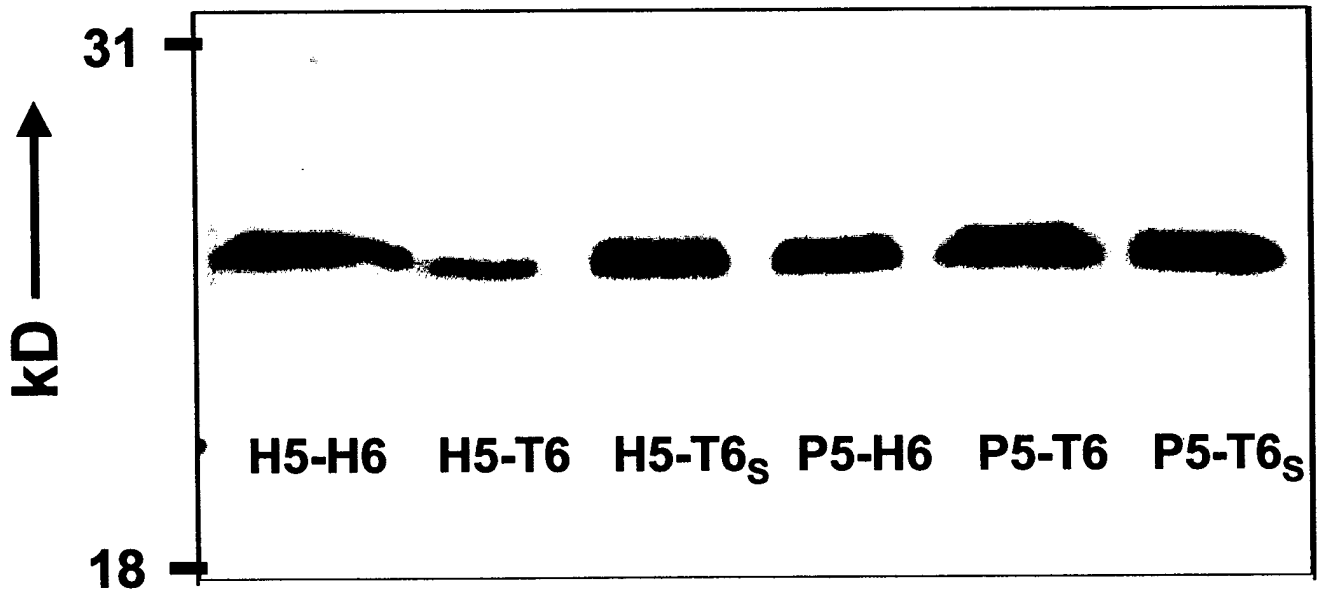

A.
Vehicle
148.94 +/- 42.86
Vehicle
125.71 +/- 42.50
0.5 μM
146.92 +/- 41.39
0.5μM
119.69 +/- 42.54
5.0 μM
101.12 +/- 26.33
5.0μM
94.41 +/- 29.71
10.0 μM
78.19 +/- 26.15
10.0 μM
69.37+/-25.83
% Mitochondrial Measurements
CMTMR Fluorescence
TMRM Fluorescence
B.
% Mean Vehicle Fluorescence
CMTMR
TMRM
FCCP Concentration (μM)


A.
H5-H6
B.
H5-T6
C.
H5-T$_D$6
D.
P5-H6
E.
P5-T6
F.
P5-T$_D$6


G.
% Mitochondrial Measurements
H5-H6
141.34 +/- 43.93
H5-T6
89.47+/-31.4
H5-T6$_D$
124.67+/-49.82
P5-H6
144.18+/-41.73
P5-T6
121.48+/-50.55
P5-T6$_D$
142.98+/-51.48
0
5
10
15
0
100
200
CMTMR Fluorescence (A/D units)
H.
% Mean H5-H6 Fluorescence
40
60
80
100
H5-H6
P5-H6
H5-T6
P5-T6
H5-T6$_D$
P5-T6$_D$

Lane	1	2	3	4	5	6
Day 5	H	.01P	H	H	H	H
Day 6	H	T	T	1.0P	T_{-9D}	T_{-9C}




31
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P5-T6S

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In Press

Propargylamines Induce Anti-Apoptotic New Protein Synthesis In Serum And NGF Withdrawn NGF-Differentiated PC12 Cells

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Number Of Words In Introduction: 618
Number Of Words In Discussion: 1497

Non Standard Abbreviations:
$\Delta\Psi_M$ – Mitochondrial Membrane Potential
ASK1 – Apoptosis Signal-Regulating Kinase 1
CCD – Charge-Coupled Device
DEP – (-)- Deprenyl
DES – (-)-Desmethyl Deprenyl
DRP – (-)-Deprenyl Related Propargylamine
GAPDH – Glyceraldehyde-3-Phosphate dehydrogenase
HBSS – Hank's Balanced Salt Solution
ISEL – In situ End Labeling
JNK – c-Jun N-terminal kinase
LCSM – Laser Confocal Scanning Microscopy
MAO-B – Monoamine Oxidase B
MAPK – Mitogen-Activated Protein Kinase
MB – Mitochondrial Buffer
MEM – Minimum Essential Medium
mfMEM – Methionine Free MEM
M/O – MEM Only
MPTP – 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
M/S – MEM with Serum Only
M/S+N – MEM with Serum and Nerve Growth Factor
NGF – Nerve Growth Factor
NB – Nuclear Buffer
PC12 - Rat Pheochromocytoma Cells
PD – Parkinson's disease
PI3-K - phosphatidylinositiol 3-kinase
PTPC – Permeability Transition Pore Complex

Recommended Section: Neuropharmacology

Abstract

(-)-Deprenyl and structurally related propargylamines increase neuronal survival independently of monoamine oxidase B inhibition, in part by decreasing apoptosis. We found that deprenyl and two other propargylamines, one which does not inhibit MAO-B, increased survival in trophically withdrawn 6 day NGF- and 9 day NGF differentiated PC12 cells but not in NGF-naïve or 3 day NGF differentiated PC12 cells. Four days of prior NGF exposure was required for the propargylamine-mediated anti-apoptosis. Studies using actinomycin D, cycloheximide and camptothecin revealed that the maintenance of both transcription and translation, particularly between 2 and 6 hours after trophic withdrawal, was required for propargylamine-mediated anti-apoptosis. Metabolic labeling of newly synthesized proteins for two dimensional protein gel autoradiography and scintillation counting showed that the propargylamines either increased or reduced the levels of new synthesis or induced de novo synthesis of a number of different proteins, most notably proteins in the mitochondrial and nuclear subfractions. Western blotting for whole cell or subcellular fraction lysates showed that the timing of new protein synthesis changes or subcellular re-distribution of apoptosis-related proteins induced by the propargylamines were appropriate to anti-apoptosis. The apoptosis-related proteins included superoxide dismutases (SOD1 and SOD2), glutathione peroxidase, c-JUN, and glyceraldehyde-3-phosphate dehydrogenase. Most notable were the prevention of apoptotic decreases in BCL-2 levels and increases in mitochondrial BAX levels. In general, DRPs appear to reduce apoptosis by altering the levels or subcellular localization of proteins that affect mitochondrial membrane permeability, scavenge oxidative radicals or participate in specific apoptosis signaling pathways.

Introduction

The propargylamine, (-)-deprenyl (DEP) inhibits monoamine oxidase B (MAO-B). DEP was first shown to reduce the death of primate nigrostriatal dopaminergic neurons exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Cohen et al., 1984) and to slow the clinical progress of human Parkinson's Disease (PD) (Parkinson, 1993). Both actions appeared to depend on MAO-B inhibition. Subsequently, DEP and deprenyl-related propargylamines (DRPs) were demonstrated to reduce neuronal loss independently of MAO-B inhibition in a variety of experimental models including cortical catecholaminergic neurons exposed to DSP-4, murine or primate substantia nigra dopaminergic neurons exposed MPTP, rat facial motoneurons after axotomy, dopaminergic cells treated with the 1-methyl-4-phenylpyridinium ion (MPP+) or nitric oxide, and hippocampal neurons exposed to kainate (see (Tatton et al., 2000) for details and references). The MAO-B independent increases in neuronal survival by DRPs were shown to involve decreased apoptosis in a number of the above models (e.g. kainate exposed hippocampal neurons, nitric oxide or MPP^+ treated dopaminergic cells). DRP anti-apoptosis also has been found in other models including partially NGF differentiated PC12 cells after serum and NGF withdrawal, rat hippocampal neurons after ischemia/hypoxia, neuroblastoma cells treated with rotenone, rat cerebellar granule neurons exposed to cytosine arabinoside, serum deprived human melanoma cells, rat retinal neurons after hypoxia, ischemia or serum withdrawal, and rat hippocampal neurons, cerebellar neurons, or neuroblastoma cells exposed to exposed to okadaic acid (also see (Tatton et al., 2000) for details and references). Notably, DRPs do not reduce all forms of apoptosis as has been shown in NGF-naïve PC12 cells (Vaglini et al., 1996), thymocytes treated with dexamethasone (Fang et al., 1995) and cerebellar granule neurons exposed to low media K^+ levels (Paterson et al., 1998).

The mechanisms of DRP anti-apoptosis are not known. DRPs bind to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Kragten et al., 1998; Carlile et al., 2000) and prevents the GAPDH upregulation essential to some forms of neuronal apoptosis (see (Tatton et al., 2000) for a review). Whether or how DRP binding to GAPDH results in decreased apoptosis is not known. DRP anti-apoptosis can require new protein synthesis (Tatton et al., 1994). In keeping with a protein synthesis dependent mechanism, DEP has been shown to alter the levels, message expression or activity of a variety of different proteins, each in a different cell type (see (Tatton et al., 2000) for references and details). Although some of the protein alterations may contribute to anti-apoptosis, the extent of DRP induced anti-apoptotic protein synthesis is not known or whether any protein synthesis changes are appropriately timed to interrupt apoptosis signaling. DRPs can prevent mitochondrial membrane potential ($\Delta\Psi_M$) dissipation in some forms of apoptosis in which $\Delta\Psi_M$ dissipation results from increases in mitochondrial membrane permeability (Paterson et al., 1998; Wadia et al., 1998; Zhang et al., 1999). In

some forms of apoptosis, increases in mitochondrial membrane permeability allow the release of mitochondrial factors that signal for apoptotic degradation (Jacotot et al., 1999). Accordingly, DRPs may alter the synthesis of proteins that influence mitochondrial membrane permeability.

We therefore examined the effects of three DRPs, DEP and desmethyldeprenyl (DES), both which inhibit MAO-B and CGP3466, which does not inhibit MAO-B (see (Waldmeier et al., 2000) for a review of CGP3466) in trophically withdrawn PC12 cells that were previously exposed to NGF for varying periods. We found show the DRPs reduced apoptosis in PC12 cells exposed to NGF or 4 days or more but not in NGF-naïve PC12 cells. The decreased apoptosis required new protein synthesis and involved alterations in the expression of a number of proteins. Proteins in the mitochondrial and nuclear fractions were most markedly involved, including proteins known to scavenge oxidative radicals and those that can alter mitochondrial membrane permeability. The time course of the changes in expression was appropriate to anti-apoptosis.

Materials and Methods

Cell Culture, Treatment And Counting. PC12 cells (ATCC, Manassas, MD) were propagated in minimum essential medium (MEM) containing 10 % horse serum, 5 % fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (MEM with serum, abbreviated as M/S), all purchased from Life Technologies (Rockville, MD). The cells were grown on 24 well plates (8×10^4 cells/well) for counting of intact nuclei as an estimate of survival, poly-L-lysine treated coverslips (1×10^4 cells/coverslip) for imaging with laser confocal scanning microscopy (LCSM) or 100 mm dishes (1×10^6 cells/plate) for protein chemistry. The cells were differentiated for up to 9 days in M/S supplemented with 100 ng/ml 7S NGF (Upstate Biotech., Lake Placid, NY). MEM with serum and NGF is abbreviated as M/S+N (see (Tatton et al., 1994; Wadia et al., 1998; Carlile et al., 2000) for further details of culture and treatment). Following incubation for 1 to 9 days in M/S+N, cells underwent three successive washes in Hanks' Balanced Salt Solution (HBSS; Life Technologies, Rockville, MD) to remove NGF and serum-borne trophic agents and then were replaced into M/S+N for controls or into MEM only (abbreviated as M/O) to induce apoptosis by serum and NGF withdrawal. Varying concentrations of DEP (Sigma-Aldrich, St. Louis, MO), DES (Toronto Chemical, Toronto, ON), CGP3466 (Novartis AG, Basel, Switzerland), actinomycin D, camptothecin or cycloheximide (all from Sigma-Aldrich, St. Louis, MO) were added to the M/S+N or M/O cultures at varying times to oppose apoptosis or to inhibit new protein synthesis. The NGF-naïve undifferentiated PC12 cells were maintained in M/S on plates, wells or coverslips for 6 days prior to washing to induce apoptosis by serum withdrawal. Washed cells were replaced into M/S or M/O with or without identical additives to those above.

Both cell survival and the percentages of cells with evidence of apoptotic nuclear degradation were assessed for all treatments. To estimate survival, the cells were seeded at a density of 8 x 10^4 cells/well in 24 well plates. Cells were harvested 24 hours after treatment and lysed. Intact nuclei were counted using a hemocytometer (see (Tatton et al., 1994; Wadia et al., 1998; Carlile et al., 2000) for details of treatment and counting methods). Percentages of cells with apoptotic nuclei were determined for cells grown on poly-L-lysine treated coverslips (density 1 x 10^4/coverslip). At varying times after treatment the cells were stained with the DNA binding dye YOYO-1 (Molecular Probes, Eugene, OR) to reveal chromatin condensation as a marker of apoptotic nuclear degradation (Tatton et al., 1994; Wadia et al., 1998; Carlile et al., 2000). Cells on coverslips were washed three times in PBS followed by 100% methanol incubation at -20° C for 30 seconds. The methanol was then replaced with YOYO-1 (1.5□M in PBS) for thirty minutes at room temperature. After three PBS washes, the cells on coverslips were mounted in Aquamount Gurr (EM Industries, Cincinnati OH) for LCSM imaging. The total number of YOYO-1 stained nuclei with chromatin condensation was counted on twenty-five 40X fields for each coverslip. Each field chosen by pairs of randomly generated x-y coordinates. The proportion of nuclei with chromatin condensation was expressed as a percentage of the total number of cells in each field. The values were pooled for three coverslips for each treatment and time point.

Caspase Inhibition Of Cells. Two caspase inhibitors, N-benzyloxycarbonyl-Val-Ala-Asp-fluomethylketone (Z-VAD-FMK, RBI, Natick, MA) and Ac-Asp-Glu-Val-Asp-CH (acetyl-DEVD aldehyde, RBI, Natick, MA) were used with PC12 cells that were supported with serum and NGF and those that were serum and NGF withdrawn. Both inhibitors were applied in MEM with the addition of 0.25% DMSO. Z-VAD-FMK has Ki's that show it to be a general caspase inhibitors while acetyl-DEVD aldehyde has a Ki for caspase 3 that indicates a strong predilection for that caspase (Garcia-Calvo et al., 1998).

DNA Electrophoresis. Cells grown in medium sized flasks and trophically withdrawn as above were examined for internucleosomal DNA digestion characteristic of apoptosis. At 6-18 hours after washing, 2x10^6 cells were rinsed with isotonic PBS and DNA was extracted and prepared according to the methods of Batistatou and Greene (Batistatou and Greene, 1993). The samples were incubated with 50 mg/ml DNAse free RNAse (Boehringer Mannheim Corp., Indianapolis, IN) at 37 deg. C for 30 minutes. The recovered soluble DNA was electrophoresed on a 1.2% agarose gel and blotted onto Gene Screen Plus membrane (Dupont, Boston , MA). Blots were probed with total genomic DNA digested with Sau 3A (Boerhinger Mannheim). ^{33}P-labeled probe was prepared by the random priming reaction and hybrization and washings were performed according to the manufacturers protocol.

Metabolic Labeling of Cells For 2-Dimensional Gel Electrophoresis Of Total Cell Protein. After a methionine-free MEM (mfMEM; Sigma-Aldrich, St. Louis, MO) wash, the PC12 cells were pulse-labeled for 1.5 hours with {^{35}S}-trans methionine (75 μCi/ml; Amersham, Piscataway, NJ) in the mfMEM at 37°C to label newly synthesized proteins and to estimate overall de novo protein synthesis. Cells undergoing treatment with DRPs were incubated in mfMEM with 10^{-9} M concentrations of each DRP. mfMEM was removed from the dishes and replaced with M/S+N, M/O or MEM with one of the DRPs for up to 4.5 hours. At 6 hours after the initial washing, all media were removed and the labeled PC12 cells were lysed (lysis buffer: 9.5 M urea, 2% CHAPS, 1.6% Servalyt; pH5-7, 0.4% Servalyt; pH 2-11 and 5% β-mercaptoethanol (all from Sigma-Aldrich, St. Louis, MO)) directly on tissue culture dishes, scraped and mechanically homogenized. To remove unincorporated radiolabel, the lysates were precipitated in 1 ml of 7% trichloroacetic acid with deoxycholate (Sigma-Aldrich, St. Louis, MO)(100 μg/ml) and centrifuged for 30 minutes at 15000 g. The resulting pellets were resuspended in 1 ml of 5% trichloroacetic acid, re-centrifuged and the supernatants discarded. The pellets were finally solubilized in 50 ml of the lysis buffer.

The lysate proteins were initially separated by isoelectric focusing on 0.20 cm x 15 cm cylindrical gels (4% acrylamide, 9.5 M urea, 2% CHAPS, 3.2% carrier ampholytes 5-7, and 0.75% carrier ampholytes 3-10). The proteins were then further separated according to molecular mass (second dimension) in the presence of 0.1% SDS on linear 5-15% gradients of acrylamide using a discontinuous buffer system. Gels were fixed in 10% acetic acid and 30% methanol and gently soaked with EN^{3}HANCE solution (Amersham, Piscataway, NJ) for 1 hour followed by incubation in 10% v/v glycerol in PBS for another hour and then vacuum-dried onto 3MM Whatmann filter paper. Exposure of the gel to pre-flashed MP Hyperfilm (Amersham, Piscataway, NJ) required 7 days for optimal resolution.

Scintillation Counting And 2-Dimensional Gel Electrophoresis Of Subcellular Protein Fractions
PC12 cells were grown and treated as described above, after washing in HBSS, cells were metabolically labeled as above with trans {^{35}S}-methionine label in mf MEM for 1.5 hours in M/S+N, M/O or MEM with DRPs as above. At 6 hours after the HBSS washing step, cells were harvested and homogenized in MOPS/ Sucrose/EDTA buffer (pH 7.2) with the addition of PMSF, DTT and leupeptin (Sigma-Aldrich, St. Louis, MO) to inhibit protease activity prior to homogenization. The suspension of PC12 cells in PBS were then centrifuged 4 times at 200g for 10 minutes at 4°C, the final wash being in nuclear buffer (NB) containing 10mM PIPES, pH 7.4, 10 mM KCl, 2mM MgCl2, 1 mM DTT, 10μM cytochalasin B and 1 mM PMSF (all from Sigma-Aldrich, St. Louis, MO). The cells were allowed to swell for 20 minutes and

homogenized by 30 strokes of a Dounce glass homogenizer. The resulting pellet was layered over 30% sucrose and centrifuged at 800g for 10 minutes to isolate the nuclear fraction. The final nuclear pellet was suspended in a small volume of NB containing 250 mM sucrose and frozen until further analysis was performed. The supernatants above the nuclear pellet were differentially centrifuged to isolate the mitochondrial fraction by a 4°C centrifugation at 14000g for 10 minutes. The resulting mitochondrial pellet was resuspended in mitochondrial buffer (MB) containing 250mM mannitol with 0.1% (w/v) BSA, pH 7.2, 0.5 mM EGTA and 5 mM HEPES supplemented with1μg/ml leupeptin, 1 μg/ml pepstatin A, 50 μg/ml antipain and 10 μg/ml chymostatin (all from Sigma-Aldrich, St. Louis, USA) and recentrifuged. The pellet was then suspended in a small volume of MB and stored until further processing. The supernatant resulting from the mitochondrial isolation containing enriched peroxisomes and soluble cytoplasmic proteins was then centrifuged at 100,000 g for 1 hour to pellet peroxisomal and cytoplasmic proteins. Finally, the above mitochondrial samples were then applied to metrizamide gradients (Sigma-Aldrich, St. Louis, MO) to further separate the mitochondrial proteins from those of the plasma membrane and the Golgi. Protein concentrations were determined for individual subcellular fractions using a BCA kit (Pierce, Rockford, MD) and identical amounts of subcellular proteins were analyzed by scintillation counting or by preparative gels. To demonstrate the enrichment of the subfractions equal amounts of proteins from each fraction were western blotted and probed with antibodies for nucleolin, 14-3-3 β and cytochrome oxidase (see (Carlile et al., 2000) for details and examples of our use of these methods).

Similarly prepared samples of the protein fractions were then used for scintillation counting of relative trans ^{35}S-methionine label incorporation. For scintillation counts, each sample was applied to one glass fiber filter (Ahlstrom Filtration, Mt. Holly Springs, PA), and allowed to adsorb. Each filter was then washed with 0.05 M Tris buffer (pH 7.2). Filters were analyzed using a Beckman scintillation counter using the windows open option. Counts of the individual fractions were compared to those for total protein.

For the analysis of individual protein subcellular fractions 20 μg of each fraction were loaded onto IEF gels (pI 3-10 range) and then taken through linear gradient gels in the second dimension (5%-15% denaturing gradient gels). The different subcellular fractions from the different treatment groups were transferred to PVDF sequencing membranes overnight by semi-dry transfer and then exposed to pre-flashed film for a period of 7-9 days. Individual new proteins from the different subcellular fractions and different treatment groups were then imaged and examined using Metamorph software (Universal Imaging, West Chester, PA).

Western Blots for Protein Levels. Alterations in the expression of specific proteins were examined using Western blots of protein lysates that were extracted at various times, 3, 6, 9, 12, 18, and 24 hours, after washing in HBSS and incubation in either M/S+N, M/O or MEM with DRP added. PC12 cell proteins were extracted as total soluble lysates or lysates for the nuclear, mitochondrial and cytosolic fractions (detailed methods and confirmation of fractionation purity are presented in (Carlile et al., 2000)). Briefly, the cells were treated as above, scraped from Petri dishes in cold PBS and harvested by centrifugation at 300g for 5 minutes at 4°C. The pellets were washed twice in cold PBS and resuspended in buffer containing 25 mM Hepes-KOH, pH 7.5,10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 5 μg/ml leupeptin, 5 μg/ml chymostatin, 5 μg/ml pepstatin A, 5 μg/ml aprotinin, plus 1 mM benzamidine and 250 mM sucrose (all from Sigma-Aldrich, St. Louis, MO). The cells were homogenized by 12-15 strokes of a glass Dounce homogenizer and centrifuged at 800g for 10 minutes at 4°C to pellet the nuclear fraction. The supernatants were again centrifuged at10000g for 15 minutes at 4°C. These pellets contained the mitochondrially-enriched fraction and the supernatant included the cytoplasmic fraction. Both nuclearly- and mitochondrially-enriched fractions were resuspended in 50 μl of the above buffer and frozen at -30°C. Prior to use, the samples were protein assayed by the BCA method. The protein fraction lysates (30-40 μg) were electrophoresed on either 10% or 12% SDS-polyacrylamide gels, and then transferred to nitrocellulose blotting membranes (BioRad, Hercules CA). Membranes were agitated at room temperature for 2 hours with primary antibodies (dilutions being 1:500 for anti-BCL-2 (Santa Cruz, Santa Cruz CA), 1:2000 for anti-BAX (BD Transduction Laboratories, Lexington, KY), 1:800 for anti-c-FOS (Geneka Biotech Inc., Montreal PQ), 1μg/ml anti-c-JUN (StressGen Biotech., Collegeville MA), 1: 5,000 anti-CuZn superoxide dismutase (SOD1)/Mn superoxide dismutase (SOD2) (BioDesign, Abingdon, UK), 1μg/ml tubulin (Molecular Probes, Eugene OR), 1:2000 NF-L (Sternberger Monoclonals Inc., Lutherville, PA), 1:1000 anti-GAPDH (Chemicon, Temecula, CA), 1:1000 anti-MAP-2 (Sigma-Aldrich, St. Louis, MO), 1:1200 anti-tyrosine hydroxylase (TH, Zymed, San Francisco CA), 1:100 μg/ml anti Glutathione peroxidase (MBL International, Watertown, MA)) in 3% blocking solution (Amersham, Piscataway, NJ). Following washing in Tris-buffered saline (pH 7.4), membranes were incubated for 1-2 hours in the appropriate alkaline-phosphatase or horseradish peroxidase labeled secondary antibody (Amersham, Piscataway, NJ). Reactions were visualized using either 3,3 -diaminobenzidine /0.2% H_2O_2 or Nitrotetrazolium blue/5-bromo-4-chloro-3-indolyl-phosphate (all from Sigma-Aldrich, St. Louis, MO) as substrates. All detected bands were then digitized using a CCD camera, imaged and analyzed using MetaMorph software.

Statistical Evaluation. In order to statistically evaluate the data, the individual measurements of data from different treatment groups were first analyzed using Statistica™ software (StatSoft) to carry out two-tailed independent sample t-testing. Levene's testing for homogeneity of variances showed that most pairs of samples were not homogeneous and Chi-Square evaluation of the distributions showed that most did not fit a normal distribution (see (Siegel, 1956)). Accordingly, analysis with parametric methods such as the t-test may not provide valid results. The data was rank ordered and first tested with the Kruskal-Wallis ANOVA by ranks (Siegel, 1956) using Statistica™. Post hoc analysis was carried out using Mann Whitney U testing. Both methods do not require homogeneity of variances, that the underlying distributions for the data be known or that the values are linearly related (Siegel, 1956). Kruskal Wallis ANOVA values are presented in the text and Mann Whitney U values are only presented where they differ from the Kruskal-Wallis. Asterisks in figures indicate $p < .05$.

Results

Dependence Of PC12 Survival After Protein Synthesis Inhibition On Duration Of Previous NGF Exposure. Previous studies have shown differing effects of protein synthesis inhibitors on PC12 cell survival. Those studies seemed to suggest that the duration of previous NGF exposure could determine how the inhibitors affect PC12 cell survival. Protein synthesis inhibitors have decreased survival of serum supported, NGF naïve PC12 cells (i.e. cells maintained in media with serum) (Torocsik and Szeberenyi, 2000), while they did not alter the decreased survival induced by serum withdrawal in the NGF naive cells (Rukenstein et al., 1991). In marked contrast, protein synthesis inhibitors prevented much of the decreased survival caused by serum and NGF withdrawal in PC12 cells that had been previously exposed to NGF for 12 days (Mesner et al., 1995). Alternatively, protein synthesis inhibitors did not alter the reduced survival caused by serum and NGF withdrawal in PC12 cells previously exposed to serum and NGF for 6 days (Tatton et al., 1994).

We examined cell survival after serum withdrawal in NGF naïve PC12 cells and after serum and NGF withdrawal in PC12 cells previously maintained in serum and NGF for 3, 6 or 9 days (figure 1). In the figures M/S or M/S+N indicates serum or serum and NGF supported cells respectively while M/O indicates serum or serum and NGF withdrawn cells. In the different experimental groups, mean cell losses at 24 hours after the withdrawal ranged from 56.3 to 60.5% with the losses being greater for the serum withdrawn than the serum and NGF withdrawn cells. We employed a range of concentrations of actinomycin D (0.06 – 30 μg/ml), camptothecin (0.02 – 200 μg/ml) and cycloheximide (0.01 – 100 μg/ml) to determine the effects of protein synthesis inhibition on cell survival. Figures 1A1-A3 show that actinomycin D concentrations of 1 μg/ml or greater, camptothecin concentrations of 2 μg/ml or greater or

cycloheximide concentrations of 1μg/ml or greater decreased NGF naïve PC12 cell survival whether the cells were serum supported or serum withdrawn. The decreases in survival are similar to those reported for NGF naïve PC12 cells treated with the protein synthesis inhibitor anisomycin (Torocsik and Szeberenyi, 2000).

In contrast, PC12 cells exposed to serum and NGF for 9 days showed increased survival after serum and NGF withdrawal when treated with actinomycin D concentrations of 1 to 3 μg/ml, camptothecin concentrations of 20 μg/ml or cycloheximide concentrations of 8 to 10 μg/ml (figures 1D1-D3). Those concentrations did not alter the survival of 9 day NGF differentiated PC12 cell that were serum and NGF supported while higher concentrations of the inhibitors (i.e. actinomycin D 30 μg/ml, camptothecin 200 μg/ml, cycloheximide 100 μg/ml) reduced the survival of the serum and NGF supported, 9 day NGF differentiated cells. PC12 cells exposed to serum and NGF for 3 (figures B1-B3) or 6 days (figures C1-C2) did not show any inhibitor induced alterations in the reduced survival caused by serum and NGF withdrawal except for decreases induced by the high inhibitor concentrations that decreased survival in the 9 day NGF differentiated cells.

Our studies were aimed at examining the dependency of DRP anti-apoptosis on new protein synthesis in PC12 cells that were maximally NGF differentiated. Based on the data in figures 1, we chose to examine PC12 cells that had been exposed to serum and NGF for 6 days. Although use of PC12 cells after 9 days of serum and NGF exposure would provide a greater degree of NGF differentiation, any changes in survival induced by protein synthesis inhibitors after DRP treatment would have to be subtracted or added to the increases in survival that the inhibitors induced after serum and NGF withdrawal.

DRPs Induce Concentration Dependent Survival Increases In PC12 Cells Exposed To NGF For 6 Days But Not Those Exposed To NGF For Less Than 4 Days

About 4 days of NGF exposure was required before DRPs increased the survival of serum and NGF withdrawn cells. Firstly, we found that DRPs increased the survival of 6 day NGF differentiated PC12 cells after serum and NGF withdrawal, but did not increase the survival of serum withdrawn, NGF naïve cells (figures 2A1-2A3). DEP (figure 2A1) and DES (figure 2A2) induced significant increases in survival (p's $< .05$ for MEM with DRP addition compared to M/O) over a concentration range of 10^{-5} to 10^{-11} M, with the greatest increase at 10^{-9} M. CGP3466 induced a similar survival-concentration relationship (figure 3A3) but also significantly increased survival at 10^{-13} M ($p< .05$ for MEM with CGP3466 addition compared to M/O). As shown in the same plots, the DRPs did not alter the survival of NGF-naïve PC12 cells that were maintained in MEM with serum for 6 days prior to undergoing serum

withdrawal. DRPs induced similar increases in survival in the 9 day NGF differentiated cells to those found for the 6 day NGF differentiated cells (data not shown).

Secondly, we examined the relationship between the duration of NGF exposure to the capacity of the DRPs to increase survival after serum and NGF withdrawal. The 10^{-9} M concentration of each DRP was tested on PC12 cells previously exposed to NGF for periods varying between one and nine days (figure 2B1, 2B2 and 2B3). Significant increases in survival relative to that for cells maintained in media with serum without NGF ($p<.05$) for the same periods become evident at the fourth to fifth day of exposure to NGF and continued to day 9. Accordingly, the capacity of the DRPs to increase survival after NGF and serum withdrawal requires at least 4 days of previous exposure to NGF. The plots in figures 2B1-2B3 are normalized to the mean survival for cells that underwent serum or serum and NGF withdrawal and therefore represent the percentage increase in survival induced by the DRPs, while those in figures 2A1-2A3 present the percentage survival relative to that for serum or serum and NGF supported cells. We altered the ordinate scale in figures 2B1-2B3 to percentage increase in survival since serum withdrawn cells showed a lower level of survival than serum and NGF withdrawn cells as shown in figure 1 and figures 2A1-2A3. By normalizing, the survival to mean values after serum or serum and NGF withdrawal, we are able to determine the timing of DRP induced increases in survival.

Decreased Survival After Serum and NGF Withdrawal In The 6 Day NGF Differentiated PC12 Cells Involves Apoptosis. We previously showed that partially NGF differentiated PC12 cells undergo apoptotic degradation after serum and NGF withdrawal using: 1) DNA gel electrophoresis to demonstrate nuclear DNA cleavage; 2) *in situ* DNA end labeling with Apop Tag™ or bodipy dUTP for nuclear DNA cleavage; 3) DNA staining with Hoechst 33258 or YOYO-1 for nuclear chromatin condensation; and 4) immunocytochemistry for antibodies against nuclear histones to demonstrate nuclear protein reorganization (Tatton et al., 1994; Wadia et al., 1998; Carlile et al., 2000).

The apoptotic nuclear degradation after serum and NGF withdrawal is accompanied by shrinkage of NGF induced neuron-like processes. The laser scanning confocal, interference contrast micrographs in figure 3A1 and 3B1 serve to compare the process development in PC12 cells exposed to serum for 6 days plus 12 hours versus those exposed to serum and NGF for the same period. The NGF-naïve PC12 cells were typically smaller and did not show the neuron-like process development found for the 6 day NGF differentiated cells. Serum and NGF withdrawal induced partial process retraction and blunting of processes in the 6 day NGF differentiated cells as illustrated in figure 3D1 for cells at 12 hours after serum and NGF withdrawal. The interference contrast micrographs for the serum and NGF withdrawn cell also showed membrane blebbing typical of apoptosis.

Figures 3A2 and 3B2 show typical YOYO-1 nucleic acid staining in nuclei of serum or serum and NGF supported cells respectively at 6 days plus 12 hours. Figures 3C2 and 3D2 show typical nuclei undergoing chromatin condensation at 12 hours after serum or serum and NGF withdrawal respectively. Typically, nuclei undergoing chromatin condensation were highly condensed with smooth edges that are found as a single intensely fluorescent body or as multiple intensely fluorescent bodies surrounded by condensed cytoplasm. Apoptotic nuclear shrinkage was evident on both the interference contrast and YOYO-1 fluorescence images. A relatively high proportion of NGF naïve cells were undergoing division as evidenced by YOYO-1 staining of mitotic spindles. A YOYO-1 stained naïve PC12 cell undergoing mitosis is shown in figure 3A2 for comparison to those undergoing nuclear chromatin condensation.

"Ladder" patterns could be discerned on DNA gel electrophoresis for the 6 day NGF differentiated PC12 cells after serum and NGF withdrawal. The ladders were best defined at 6 to 18 hours after serum and NGF withdrawal as illustrated in figure 3E. Finally, treatment with either a caspase 3 inhibitor (acetyl-DEVD aldehyde) or a general caspase inhibitor (ZVAD-FMK) induced concentration dependent reductions in the decreased survival caused by serum and NGF withdrawal in the 6 day NGF differentiated PC12 cells (figure 3F). ZVAD increased the survival to a maximum of 82% while DEVD increased survival to a maximum of 61%. The caspase inhibitors did not alter survival of the 6 day NGF differentiated cells when they were serum and NGF supported. These findings indicate that the apoptosis signaling induced by the serum and NGF withdrawal in the 6 day NGF differentiated PC12 cells was caspase dependent, apparently to the greatest extent for caspases other than caspase 3.

The % of YOYO-1-stained nuclei showing chromatin condensation in the 6 day NGF differentiated PC12 cells first increased above baseline at 6 hours after serum and NGF withdrawal, reaching a maximum of 12-13% approximately 12 hours after the withdrawal of serum and NGF (figure 4A1-4A3). Treatment with 10^{-9} M DEP (figure 4A1), DES (figure 4A2) or CGP3466 (figure 4A3) decreased the percentages of nuclei with chromatin condensation to less than 4% at all time points. Since nuclei with chromatin condensation exist for a limited period of time during apoptotic degradation, the numbers of condensed YOYO-1 stained nuclei at a given time point reflects only those cells in the degradative phase at the time of fixation and do not provide an estimate of the overall cell loss resulting from apoptosis.

We therefore also performed counts of intact nuclei taken from PC12 cells that were grown in wells and exposed to identical conditions as those stained with YOYO-1 on cover glasses. The counts provide a cumulative estimate of cell loss prior to a given time point and were expressed as the percentage of surviving cells compared to that found for the 0 hour time point. Survival was reduced to about 40 % of the number of serum and NGF supported cells by 24 hours after the withdrawal of serum and NGF (figure 4B1 and 4B2). The reductions in survival appeared to occur in two phases: a first phase involving

losses of 16-18% of the cells occurring over the first 4.5 to 6 hours after serum and NGF withdrawal and a second phase involving the further loss of about 45% of the cells between 6 hours and 24 hours after serum and NGF withdrawal. The approximate 9% decrease in survival that was evident at 3 hours was not accompanied by an increase in the percentage of nuclei with chromatin condensation at the same time point (compare figures 4A1-4A3 with figures 4B1-B2). The absence of an increase in cells with evidence of nuclear chromatin condensation at times of less than 6 hours suggest that the cell loss during the first phase was not apoptotic. Accordingly, the data in figures 3E and 4B1-4B2 suggests that the general caspase inhibitor rescued most, if not all, of the apoptotic cells.

The DRPs increased cell survival and slowed the rate of cell loss over phase 2 but not during phase 1 (figures 4B1 and 4B2). The findings suggest that the mechanisms underlying cell loss during the first 6 hours after serum and NGF withdrawal were not altered by the DRPs, while those operating after 6 hours were responsive to DRP treatment. Data like that in figure 4B indicates that the DRPs decrease apoptosis in phase 2 by 79 to 94%.

We progressively delayed the addition of the DRPs relative to the time of serum and NGF withdrawal in the 6 day NGF differentiated cells (figures 4C1 and 4C2). DRP addition could be delayed for 2 to 3 hours before the capacity of the DRPs to increase survival was diminished (p's $< .05$ compared to survival for adding DRPs at time 0). After delays of 4 or 5 hours or more, the DRPs did not provide any increase in survival (p's $> .05$ compared to survival for M/O). Accordingly, the maximum effect of DRPs on apoptosis caused by serum and NGF withdrawal occurs during the first two hours after their addition followed by a gradual decrease in the anti-apoptosis over hours 2 to 5. Figures 4A1 – 4A3 and our previous work (Wadia et al., 1998) indicate that nuclear apoptotic degradation begins at about 6 hours after serum and NGF withdrawal and is maximal at 12 hours. Hence the delay experiments suggest that the DRPs act relatively early in the apoptosis signaling process.

Dependency Of DRP Anti-Apoptosis On New Protein Synthesis. Metabolic labeling using ^{35}S methionine incorporation was determined for the three protein synthesis inhibitors at maximum concentrations (see figure 1C1-1C3) that did not reduce survival (actinomycin D 3 μg/ml, cycloheximide 10 μg/ml and camptothecin 20 μg/ml). Scintillation counting showed that new protein synthesis was decreased by 92% or more for each of those concentrations (bars graphs in figures 5A1, 5A2 and 5A3) when the maximum concentrations of the protein synthesis inhibitors that did not decrease survival were applied. Those concentrations of the protein synthesis inhibitors were applied to the 6 day NGF differentiated PC12 cells following serum and NGF withdrawal. Similar to figure 1, figures 5B1 shows that those concentrations of the protein synthesis inhibitors did not affect the survival of the 6 day NGF

differentiated PC12 cells when they were serum and NGF supported or after serum and NGF withdrawn (p's > .05).

Treatment with each protein synthesis inhibitor blocked or markedly reduced the increased survival afforded by the DRPs at concentrations of 10^{-7} to 10^{-11} M (figures 5B2-5B4, p's < .05). Accordingly, new protein synthesis is necessary for the anti-apoptosis provided by the three DRPs. Experiments in which the addition of the protein synthesis inhibitors were delayed relative to the withdrawal of serum and NGF and the addition of 10^{-9} M DRP showed that DRP anti-apoptosis progressively decreased for inhibitor addition delays beyond 3 to 7 hours (figures 5C1 and 5C2). The delay curves for DEP (figure C1) were shifted to about 2 hour longer times compared to those for DES (figure C2). Delay curves similar to those for DES were found for CGP3466 (data not shown).

DRPs Alter The New Synthesis Of A Number Of Proteins During Apoptosis. We examined metabolically labeled 2D protein gels for whole cell lysates at 6 hours after washing. Six hours represents the end of the first phase of decreased survival, which corresponded to the onset of increased apoptotic nuclear degradation revealed by chromatin condensation and the time at which delays in the addition of protein synthesis inhibitors allowed for maximal increases in survival to be induced by the DRPs (figures 5B1 and 5B2). Autoradiograms like those in figure 6 represent the incorporation of ^{35}S methionine into proteins and hence provided an estimate of the synthesis of new proteins during the first 6 hours after washing. The partially NGF differentiated PC12 cells maintained in serum and NGF after washing were found to incorporate the ^{35}S label into a relatively large number of proteins as indicated by the distribution and number of punctate autoradiographic densities (figure 6A1). In contrast, relatively fewer densities were detected in samples taken from cells that were NGF and serum withdrawn (figure 6A2). As well as the overall decrease in densities, a number of densities were evident in serum and NGF withdrawn cells that did not appear to be present in the serum and NGF supported cells (compare figures 6A1 and 6A2). The autoradiograms also appeared to show that DRP treatment at 10^{-9} M increased the number of densities in the serum and NGF withdrawn cells (compare figure 6A2 to 6A3). The autoradiograms were repeated for three experiments, each of which offered similar changes in the numbers and distributions of densities for labeled proteins. The DRP induced increase in densities appeared to result from the recovery of some densities lost with serum and NGF withdrawal together with the appearance of other densities that were not evident in either serum and NGF supported or serum and NGF withdrawn cells.

Scintillation counting for ^{35}S in the whole cell lysates appeared to support the autoradiographic results. New protein synthesis for the serum and NGF withdrawn cells at 6 hours (M/O in figure 6C) was reduced to 29.8+/-3.4 % of that for cells which were washed and replaced in media with serum and NGF

(M/S+N at 6 hours, $p < .001$ for M/O compared to M/S+N), while treatment of the serum and NGF withdrawn cells with 10^{-9} M DEP resulted in a recovery to 75.8+/- 4.7% of that for cells which were washed and maintained in media with serum and NGF ($p < .001$ compared to M/O).

Evidence that the DRPs differentially affected new protein synthesis in different subcellular protein fractions was found with both 2D autoradiograms and scintillation counting (figures 6B1-6B4 and 6C). 2D gel autoradiograms for the plasma membrane (figure 6B1-i to 6B1-iii), mitochondrial (figure 6B2-i to 6B2-iii), cytosolic (figure 6B3-i to 6B3-iii) and nuclear fractions (figure 6B4-i to 6B4-iii) revealed overall decreases in autordiographic densities for serum and NGF withdrawn cells at 6 hours. The DRPs induced clear changes in densities on the 2D gel autoradiograms for the mitochondrial (figure 6B2-iii) and nuclear fractions (figure 6B4-iii). For example, treatment with 10^{-9} M DEP induced recovery of some densities in all subfractions, most notably in the mitochondrial subfraction. Like those for the total protein pool, the autoradiograms for the subfractions indicated that the DRPs induced the recovery of some densities lost with serum and NGF withdrawal but also induced the disappearance of some densities evident in either serum and NGF supported or serum and NGF withdrawn cells. The autoradiograms also suggested that the DRPs induced the synthesis of some novel proteins that were not newly synthesized in either serum and NGF supported or serum and NGF withdrawn cells or that the DRPs induced the subcellular movement of newly synthesized proteins from one subcellular fraction to another. The appearance of novel densities appeared most evident in the mitochondrial fraction (compare 6B2-iii to 6B2-i and 6B2-ii).

Figure 6C also shows the scintillation counts for the subfractions. New protein synthesis in the plasma membrane fraction was reduced from 25.9 +/- 8.1 % of total new protein in serum and NGF supported cells to less than 0.4 +/- 0.2 in serum and NGF withdrawn cells ($p < .001$ for M/O compared to M/S+N). DEP, or the other DRPs, did not significantly increase the labeled protein in the plasma membrane fraction (1.2 +/- 0.8, $p > .05$ for M/O compared to MEM with DEP). The reduction in new protein synthesis after serum and NGF withdrawal was less marked in the cytoplasmic fraction (19.7+/- 0.4 % of total labeled protein for M/S+N and 7.0+/-1.5 % for M/O, $p < .01$ for M/S+N compared to M/O). DEP did not significantly increase the counts for the cytoplasmic fraction (10.1+/-1.4 %, $p > .05$ for M/O compared to MEM with DEP). Serum and NGF withdrawal markedly reduced new protein synthesis in the mitochondrial fraction (25.1 +/- 1.7 % of total labeled protein for M/S+N and 1.2 +/- 0.7 % for M/O, $p < .01$ for M/S+N compared to M/O) and in the nuclear fraction (21.5 +/- 1.6 % of total labeled protein for M/S+N and 3.5 +/- 1.6% for M/O, $p < .01$ for M/S+N compared to M/O). DEP treatment induced levels of new protein synthesis for the mitochondrial and nuclear fractions in the serum and NGF withdrawn cells that were not significantly reduced from those for serum and NGF supported cells (33.6+/-5.7 % for the mitochondrial fraction and 23.6+/-4.2 % for the nuclear fraction, p's >.05 for M/O with DEP

compared to M/S+N). Accordingly, DEP treatment returned the levels of newly synthesized protein in the mitochondrial and nuclear fractions to the levels found in serum and NGF supported cells.

Proteins Affected By DRPs. Western blots for multiple time points were performed to identify some of the proteins whose synthesis was altered by DRP treatment. Examples of typical western blots are presented in figure 7, while optical density measurements taken from western blot bands are presented in figure 8. Figures 7A1, 7A2, 7B1 and 7B2 show typical changes in BCL-2 immunoreaction induced by DES or DEP at 1, 3, 6 and 9 hours or 3, 6, 12 and 24 hours respectively following serum and NGF withdrawal. Levels of BCL-2 immunoreaction were reduced by 3 hours following serum and NGF withdrawal, with a further decline in protein levels over subsequent hours (figures 7A2, 7B1 and 8A). DEP (figure 7B2), DES (figure 7A2) and CGP3466 (not shown) at 10^{-9} M maintained BCL-2 immunoreaction at levels similar to those found in serum and NGF supported cells (figure 8A). Notably, the DRPs did not alter levels of BCL-2 immunoreaction in cells that were maintained in M/S+N after washing and therefore were not entering apoptosis (see right hand four bands in figure 7A1 for an example).

Proteins like Cu-Zn superoxide dismutase (SOD1, figure 8C), Mn superoxide dismutase (SOD2, figure 8D), glutathione peroxidase (Glut. Perox., figure 8E) and tyrosine hydroxylase (TH, figure 8H) showed progressive decreases after serum and NGF withdrawal that were similar to those found for BCL-2. They were maintained at serum and NGF supported levels or were increased above those levels by DRP treatment.

Several proteins including c-FOS (figure 8F), c-JUN (figures 7C1-1, 7C1-2, 7C2-1, 7C2-2 and 8G) and GAPDH (figure 8I and also see (Carlile et al., 2000)) showed increased immunoreaction after serum and NGF withdrawal that were decreased by DRP treatment. c-JUN underwent a rapid transient increase that was only detected at the 3 hour time point. Two examples of western blots for c-JUN are presented in figures 7C1 and 7C2 to demonstrate the reproducibility of the transient c-JUN increase and its disappearance with DRP treatment.

Four proteins that we examined did not show any differences in immunoreaction for the whole cell lysates with DRP treatment: BAX (figure 8B), neurofilament light protein (NFL, figure 8J), microtubule associated protein 2 (MAP-2, figures 7D1, 7D2 and 8K) and α-tubulin (figure 8L). Although BAX levels for the whole cell lysates did not decrease after serum and NGF withdrawal, we found differences in BAX immunoreaction in nuclear, mitochondrial and cytosolic fractions after serum and NGF withdrawal and following DRP treatment. In cells that were washed and then maintained in media with serum and NGF, BAX immunoreaction was more strongly evident in the nuclear and cytosolic fractions than in the mitochondrial fraction (upper panel in figure 7E labeled M/S+N). BAX

immunoreaction progressively decreased in the cytosolic and nuclear fractions but was increased in the mitochondrial fraction by 3 hours after serum and NGF withdrawal (middle panel in figure 7E labeled M/O). DRP treatment prevented or reduced the decreased immunoreaction in the nuclear and cytosolic fractions and also appeared to reduce the increased BAX immunoreaction in the mitochondrial fraction after serum and NGF withdrawal (figure 7E, lower panel labeled M/O + DES).

Discussion

NGF induces multiple changes in PC12 cells including: 1) differentiation into an action potential generating, neurite-bearing, sympathetic neuron-like phenotype; 2) a reduction in proliferation; and 3) an increase in survival (Greene and Tischler, 1982). Similar to previous reports, we found that DEP does not decrease apoptosis caused by serum withdrawal from NGF-naive PC12 cells (Vaglini et al., 1996). We found that four days of prior NGF exposure is necessary before DRPs can reduce PC12 cell apoptosis initiated by serum and NGF withdrawal.

DEP was first shown to prevent decreases in dopaminergic indices of nigrostriatal neuronal survival caused by MPTP exposure in primates (Cohen et al., 1984), which was interpreted to show that DEP protected the neurons by blocking MPTP conversion to MPP^+ by MAO-B. A number of clinical trials showed that DEP can slow the clinical progression of PD (see (Parkinson, 1993) as an example), but it is uncertain whether the slowing represents reduced neuronal death or alterations in dopamine metabolism. It is certain that DEP and other DRPs reduce neuronal death induced *in vivo* and *in vitro* by a wide variety of insults in a number of different neuronal models. Those insults have included 6-hydroxydopamine, MPP^+, MPTP, nitric oxide or peroxynitrite, DSP-4, glutathione depletion, peripheral nerve crush or axotomy, optic nerve crush, hypoxia and/or ischemia, cytosine arabinoside, excitotoxins, trophic insufficiency, thiamine deficiency, okadaic acid and aging. The neurons or neuron-like cells have included mesencephalic or nigral dopaminergic neurons, hippocampal neurons, dentate neurons, cerebellar granule and Purkinje neurons, cerebral cortical neurons, thalamic neurons, retinal ganglion neurons, spinal and facial motoneurons, neuroblastoma cells, and partially differentiated PC12 cells (reviewed in (Tatton et al., 2000)). Studies involving a range of models have shown that DRPs can increase neuronal survival without MAO-B inhibition and by reducing apoptosis (Tatton et al., 2000). Since PC12 cells do not express MAO-B (Youdim et al., 1986) and CGP3466 does not inhibit MAO-B (Kragten et al., 1998; Waldmeier et al., 2000), DRP anti-apoptosis in the serum and NGF withdrawn, 6 day NGF differentiated PC12 cells is also MAO-B independent.

As previously, we examined multiple indices to determine whether the increases in cell survival result from anti-apoptosis. Time course studies of survival versus the percentage cells with nuclear chromatin condensation showed that about 16% of the cells die during the first 6 hours after serum and

NGF withdrawal by a process that is unlikely to be apoptotic. The remaining cell loss, occurring after 6 hours, meets multiple criteria for apoptosis and is responsive to DRP treatment. ZVAD-FMK a general caspase inhibitor prevented almost 100% of the apoptosis while acetyl DEVD aldehyde, a caspase 3 inhibitor prevented less than 50%. This is in keeping with previous studies of NGF differentiated PC12 cells, which have shown that general caspase or caspase 2 inhibitors almost completely prevent apoptosis initiated by NGF or serum and NGF withdrawal while caspase 3 inhibition only partially prevents the apoptosis (Haviv et al., 1997; Haviv et al., 1998; Stefanis et al., 1998).

We found that new protein synthesis is necessary for reductions in apoptosis induced by the three DRPs. In various models, protein synthesis inhibitors can slow or reduce apoptosis, induce apoptosis or may not affect the extent or timing of apoptosis (Eastman, 1993). Hence the use of protein synthesis inhibitors to examine anti-apoptotic agents can be complicated if the apoptosis itself requires new protein synthesis or if a protein synthesis inhibitor induces apoptosis. Apoptosis initiated by serum withdrawal from NGF-naïve PC12 cells (Rukenstein et al., 1991; Mesner et al., 1995) or serum and NGF withdrawal from partially NGF differentiated PC12 cells (Tatton et al., 1994) are not new protein synthesis dependent. Alternatively, apoptosis initiated in fully differentiated PC12 cells by NGF withdrawal requires new protein synthesis (Mesner et al., 1995). Some protein synthesis inhibitors induce apoptosis over one concentration range and reduce apoptosis over another (Torocsik and Szeberenyi, 2000). Our findings suggest that the duration of previous exposure to NGF determines how protein synthesis inhibitors influence apoptosis signaling initiated by serum and NGF withdrawal in PC12 cells.

Our experiments in which protein synthesis inhibitor addition was delayed relative to serum and NGF withdrawal and DRP addition suggested that critical anti-apoptotic alterations in transcription/translation induced by DES begin prior to 3 hours after DES addition while those for DEP continue to 5 hours. DES is a principal metabolite of DEP (Baker et al., 1999) and studies using P450 inhibitors suggest that DEP anti-apoptosis requires DEP metabolism to DES (Tatton and Chalmers-Redman, 1996). The relative prolongation of the protein synthesis inhibitor blockade of DEP anti-apoptosis may reflect a period necessary for DEP metabolism to DES. The experiments in which DRP addition was delayed relative to the onset of serum and NGF withdrawal indicate that the apoptosis signaling events that are critical to DRP anti-apoptosis occur between 2 and 5 hours after serum and NGF withdrawal, which is within the same time domain for critical DRP induced new protein synthesis suggested by the protein synthesis inhibitor delay experiments.

Metabolic labeling suggested that DRPs induced changes in the new synthesis of a number of proteins with the most marked changes involving the mitochondrial and nuclear protein subfractions. Previous work used differential display PCR to identify four genes, c-jun, heat-shock protein 70, phosphoglycerate kinase, and calpactin I heavy chain, whose expression was increased in retinal ganglion

neurons initiated into apoptosis by serum deprivation or hypoxia (Xu et al., 1999). In that study, 10^{-9} M DEP reversed the increases in c-jun and heat-shock protein 70 gene expression but not that for the other genes. Like the retinal study, we have found that DRPs alter the synthesis of some proteins but not others and found a transient increase in c-JUN. As well as c-JUN, we found alterations for a number of proteins that previously were shown to play a role in PC12 cell apoptosis, including BCL-2, BAX, SOD1, SOD2, c-FOS, glutathione peroxidase and GAPDH.

Importantly, we found that the timing of alterations in the levels or subcellular distribution of those proteins induced by DRPs were appropriate to anti-apoptosis. In this and previous studies (Wadia et al., 1998; Carlile et al., 2000), we found that apoptotic nuclear degradation first became evident at about 6 hours after serum and NGF withdrawal. Western blots showed that the levels of anti-apoptotic proteins decreased and those of pro-apoptotic proteins increased by 3 hours after serum and NGF withdrawal. DRP treatment caused those early alterations to return toward control levels in association with a decrease in the percentage of cells with apoptotic nuclear degradation. The timing for changes in the levels of apoptotic and anti-apoptotic proteins appear in accord with that found by the protein synthesis inhibition and DRP addition delay experiments.

Increases in the phosphorylation and levels of c-JUN are induced by NGF withdrawal from sympathetic neurons or NGF differentiated PC12 cells and involves the upregulation of c-Jun N-terminal kinase (JNK) and/or p38 MAPK (Xia et al., 1995; Maroney et al., 1999). Pharmacological inhibition of JNK blocks apoptosis in NGF differentiated PC12 cells induced by NGF withdrawal but not apoptosis induced in NGF-naïve PC12 cells induced by serum withdrawal (Maroney et al., 1999). NGF withdrawal in 6 day NGF differentiated PC12 cells induced an increase in the levels of an endogenous MAPK kinase kinase, apoptosis signal-regulating kinase 1 (ASK1) (Kanamoto et al., 2000). ASK1 upregulation was necessary for c-JUN upregulation and apoptosis initiated by NGF withdrawal in the cells (Kanamoto et al., 2000). The timing of the c-JUN upregulation was consistent with the transient c-JUN increase that we found 3 hours after serum and NGF withdrawal.

The prevention of decreases in BCL-2 and the decreased mitochondrial BAX localization induced by the DRPs may contribute to the maintenance of $\Delta\Psi_M$ found with DRP treatment of neurons or neuron-like cells entering apoptosis (Paterson et al., 1998; Wadia et al., 1998). BCL-2 prevents decreases in $\Delta\Psi_M$ caused by agents that increase mitochondrial membrane permeability and induce apoptosis in PC12 cells (Dispersyn et al., 1999). Mitochondrial BAX accumulation, similar to our finding in the NGF differentiated PC12 cells, has been shown for a number of forms of apoptosis including NGF withdrawal from sympathetic neurons (Putcha et al., 2000). Mitochondrial BAX accumulation increases mitochondrial membrane permeability and decreases $\Delta\Psi_M$ (Narita et al.,1998). Prevention of increased mitochondrial membrane permeability can be a key step in blocking apoptotic degradation (Jacotot et

al.,1999).

DRPs have been shown to bind to GAPDH in association with reductions in apoptosis (Kragten et al., 1998; Carlile et al., 2000). Upregulation of GAPDH together with the dense nuclear accumulation of GAPDH immunoreactivity are characteristic of apoptosis that can be blocked by GAPDH antisense oligonucleotides (reviewed in (Tatton et al., 2000)). The tumor suppressor protein, p53, has been shown to upregulate GAPDH in neuronal apoptosis (Chen et al., 1999). p53 activation is downstream to JNK activation but upstream to BAX (Aloyz et al., 1998; Mielke and Herdegen, 2000) in a variety of apoptosis models, which may suggest that DRP binding to GAPDH could uncouple a JNK-p53-GAPDH apoptosis signaling pathway.

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Figure Legends

Figure 1. Effect Of Protein Synthesis Inhibitors On PC12 Survival Depends On Previous NGF Exposure. Varying concentrations of actinomycin D (**A1, B1, C1 and D1**), camptothecin (**A2, B2, C2 and D2**), and cycloheximide (**A3, B3, C3 and D3**) were applied to NGF naive PC12 cells (**A1-A3**) or PC12 cells previously exposed to NGF for 3 (**B1-B3**), 6 (**C1-C3**) or 9 days (**D1-D3**) to determine whether the duration of previous NGF exposure altered the effect of protein synthesis inhibition on cell survival. Cells were either trophically supported by serum or serum and NGF (open circles) or trophically withdrawn (filled circles). Values are means +/- SEMs in all figures. Means are for 8 to 16 measurements in this and other plots of survival. Distance between dotted lines show the extent of control SEMs for trophically supported (labeled M/S or M/S+N) or trophically withdrawn (M/O) survival without protein inhibitor treatment. Asterixes indicate that values significantly differ from controls ($p' < .05$).

Figure 2. Concentration Relations For DRP Increases In Survival And Duration of NGF Exposure Necessary For DRP Induced Increases In Survival After Serum And NGF Withdrawal. A1, A2 and A3 show that DEP, DES and CGP3466 concentrations of 10^{-5} to 10^{-11} M increase the survival of 6 day NGF differentiated PC12 cells after serum and NGF withdrawal but similar concentrations do not increase the survival of NGF naïve cells after serum withdrawal. **B1, B2 and B3** show that approximately 4 days of prior NGF exposure is necessary for the three propargylamines to increase the survival of PC12 cells after serum and NGF withdrawal. Asterixes indicate significant increases ($p<.05$) in survival relative to those for M/O.

Figure 3. Apoptotic Markers In NGF-Naive and 6 Day NGF Differentiated PC12 Cells. A1-D2. Each horizontal pair of laser confocal scanning micrographs is for an identical image field. The left hand member presents an interference contrast image and the right hand image presents YOYO-1 fluorescence. **A1-A2 and B1 - B2** are for NGF-naive PC12 cells maintained in M/S for 6 days and 6 Day NGF differentiated PC12 cells respectively that were washed repeatedly and replaced into M/S or M/S+N to maintain trophic support. **C1- C2 and D1 - D2** are for NGF-naive PC12 cells maintained in M/S for 6 days and 6 day NGF differentiated PC12 cells respectively that were trophically withdrawn by repeated washing and placement into M/O. The interference contrast images for 6 day NGF differentiated PC12 cells show apoptotic membrane blebbing and process withdrawal (compare B1 and D1). The filled arrows indicate nuclei with chromatin condensation while the open arrow indicates a nucleus undergoing mitosis for comparison. **E.** DNA electrophoresis gels showing laddering typical of internucleosomal DNA digestion at 6 – 18 hours after serum and NGF withdrawal in 6 Day NGF differentiated PC12 cells. **F.** DEVD, a caspase inhibitor with a predilection for caspase 3, and ZVAD-FMK, a general caspase

inhibitor, induce about 45% and 65% reductions respectively in the decreased survival induced by serum and NGF withdrawal in 6 day NGF differentiated PC12 cells. Asterixes indicate significant increases in survival ($p > .05$) compared to cells not treated with a caspase inhibitor (0 μM concentration).

Figure 4. DRP Alterations In The Time Courses Of Appearance Of Nuclear Chromatin Condensation And Decreased Survival Initiated In NGF Differentiated PC12 Cells By Serum And NGF Withdrawal. A1, A2 and A3 show the time course of the appearance of nuclei with chromatin condensation shown by YOYO-1 staining in 6 day NGF differentiated PC12 cells and the time course of reductions in nuclei with chromatin condensation induced by 10^{-9} M DEP, DES or CGP3466 respectively. **B1 and B2** show the time course of decreased 6 day NGF differentiated PC12 cell survival initiated by serum and NGF withdrawal and the time course of the increased survival induced by 10^{-9} M DEP, DES or CGP3466 after serum and NGF withdrawal. **C1 and C2** show that delays in the administration of 10^{-9} M DEP or DES administration respectively of less than 2 hours did not alter the increase in survival induced by the DRPs while delays in administration of 3 to 5 hours progressively reduced the capacity of the DRPs to increase survival of the 6 day NGF differentiated PC12 cells. Asterixes indicate significant increases in survival ($p > .05$) compared to M/O.

Figure 5. Transcriptional Or Translational Inhibition Prevents DRP Anti-Apoptosis In NGF Differentiated PC12 Cells After Serum And NGF Withdrawal. A1., A2. and A3. show that 3 μg/ml, of actinomycin D, 20 μg/ml of camptothecin or 8 μg/ml of cycloheximide respectively, which do not reduce the survival of 6 day NGF differentiated PC12 cells, reduce new protein synthesis by 94% or more (black bars – serum + NGF supported, gray bars – serum + NGF withdrawn) . Employment of the three protein synthesis inhibitor at those concentrations did not alter the survival of serum and NGF supported or serum and NGF withdrawn cells (**B1**) but prevented the three DRPs administered at 10^{-7}, 10^{-9} and 10^{-11} M (**B2, B3 and B4**) from increasing survival after serum and NGF withdrawal. Progressive delays in the administration of 3 μg/ml actinomycin D (**C1**) or 8 μg/ml of cycloheximide (**C2**) showed that the protein synthesis inhibitors completely blocked the capacity of 10^{-9} M DEP from increasing survival for delays of up to 5 hours and 10^{-9} M DES for delays of up to 3 hours. Longer delays in the administration of the two inhibitors allowed for progressive increases in survival. Asterixes indicate significant increases in survival ($p > .05$) compared to M/O.

Figure 6. DEP Induces Widespread Changes In New Protein Synthesis, Notably In Nuclear And Mitochondrial Proteins, In 6 Day NGF Differentiated PC12 Cells Entering Apoptosis After Serum and NGF Withdrawal. Typical autoradiograms for 2D protein gels for metabolically labeled total protein at 6 hours after washing and replacement in media with serum and NGF (**A1**), washing and placement in media without serum or NGF (**A2**), and washing and placement in media without serum or NGF with 10^{-9} M DEP (**A3**). **B1-i to B1-iii, B2-i to B2-iii, B3-i to B3-iii and B4-i to B4-iii** present 2D autoradiograms for the plasma membrane, mitochondrial, cytosolic and nuclear protein subfractions respectively at 6 hours after washing and replacement in serum and NGF (**i panels**), washing and placement in MEM only (**ii panels**), and washing and placement in MEM with 10^{-9} M DEP (**iii panels**). **C** presents scintillation counts for metabolically labeled proteins corresponding to those for total protein and each of the subcellular fractions. Pl.M., Mit., Cyt. and Nuc. indicate plasma membrane, mitochondrial, cytosolic and nuclear subfractions respectively. Asterixes above bars indicate significant increases in counts ($p > .05$) compared to those for M/O.

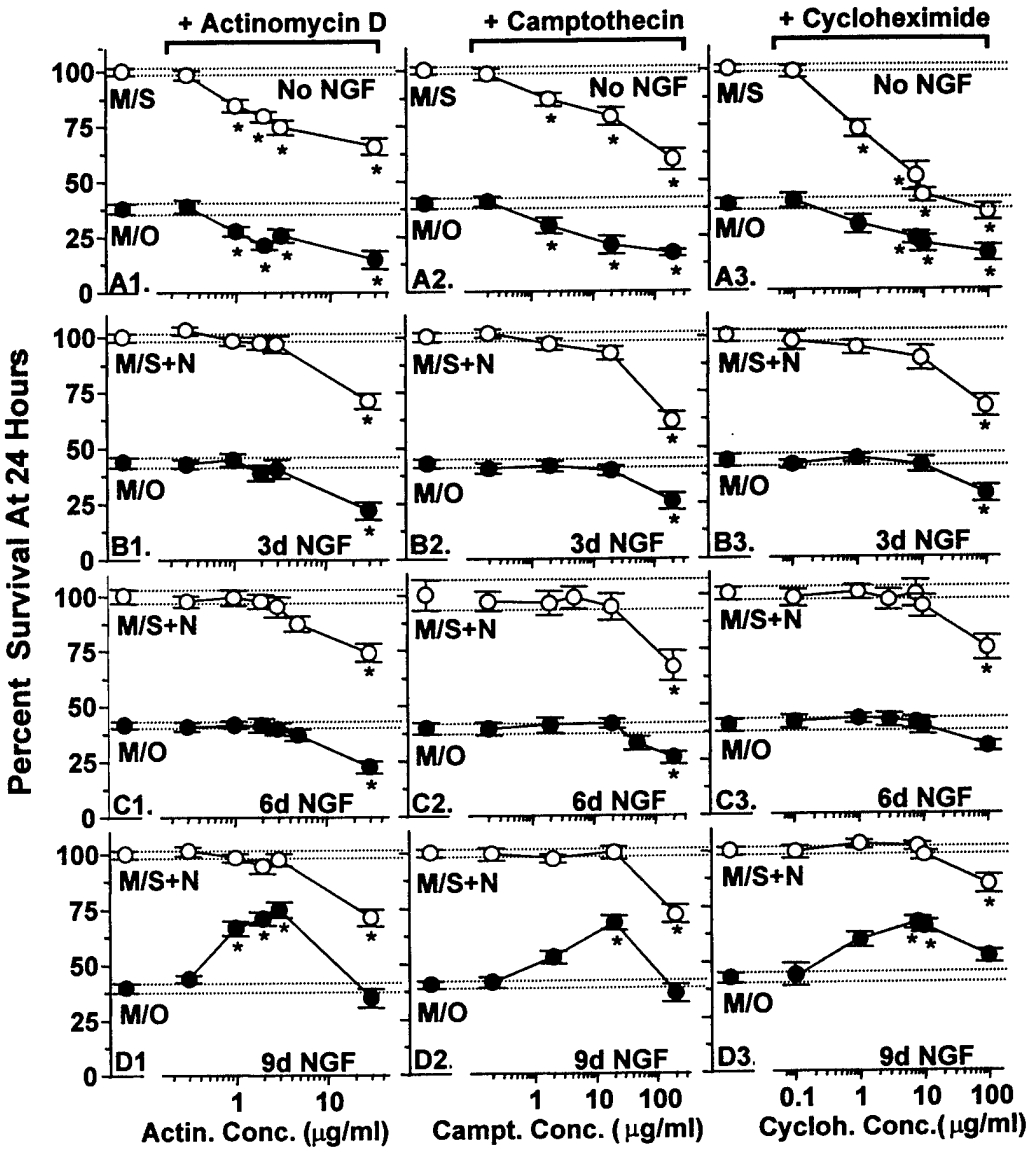
Figure 7. Western Blots For The Immunodensity Of Selected Proteins After Serum And NGF Withdrawal With and Without DRP Treatment. BCL-2 immunodensity at 1, 3, 6 and 9 hours after washing and replacement in media with serum and NGF support (**A1**) or placement into media without serum and NGF (**A2**). Immunodensity for BCL-2 (**B1-B3**), c-JUN (**C1-1, C1-2, C2-1 and C2-2**), and MAP-2 (**D1 and D2**). **SN6-** 6 hours after washing and replacement in media with serum and NGF support; **O3, O6, O12 and O24** – 3, 6, 12 and 24 hours respectively after washing and placement into media without serum and NGF; and **d3, d6, d12 and d24** - 3, 6, 12 and 24 hours respectively after washing and placement into media without serum and NGF with added 10^{-9} M DEP. **E.** BAX immunodensity for nuclear, mitochondrial and cytosolic subfractions at 3, 6, 9 and 12 hours after washing and replacement in media with serum and NGF support (**upper panel**), after washing and placement into media without serum and NGF (**middle panel**), and after washing and placement into media without serum and NGF with added 10^{-9} M DES (**lower panel**).

Figure 8. Plots Of Average Western Blot Immunodensity Above Background For Selected Proteins At 0, 3, 6, 12 and 24 Hours After Serum and NGF Withdrawal. Each point represents the average +/- SEM optical density for immunodensity bands from 3 experiments and is normalized against the average optical density for bands from serum and NGF supported, 6 day NGF differentiated PC12 cells at 6 hours after washing. The values for serum and NGF supported, 6 day NGF differentiated PC12 cells are plotted at time 0. Other points represent the time after washing for serum and NGF withdrawn cells. Each plot is

labeled for the respective primary antibody. A 9-hour time point was substituted for the 12 hour time point for GAPDH.

Acknowledgements

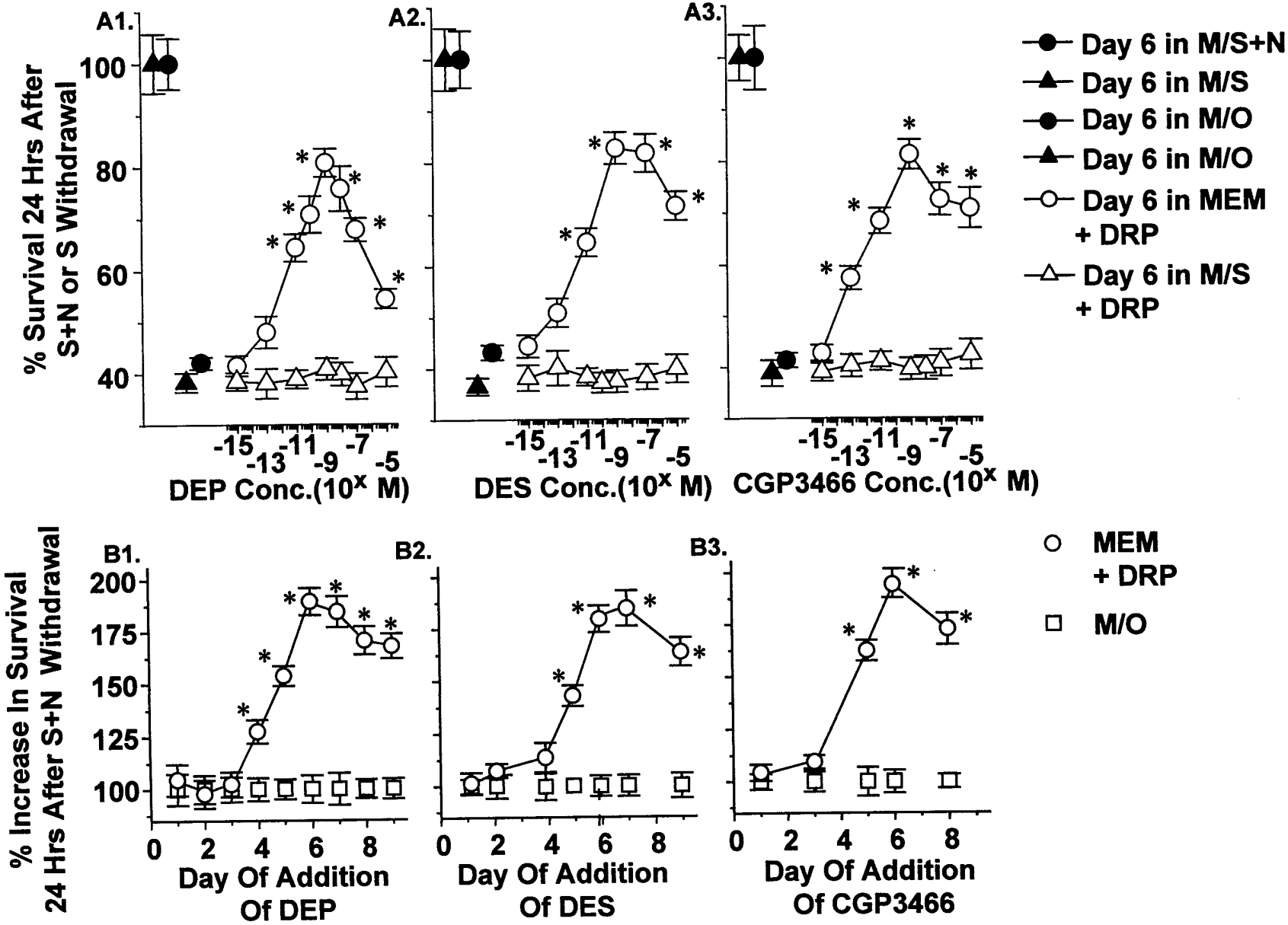
This research was supported by a grant from the Lowenstein Foundation and US Army Grant PSA280. Novartis Pharmaceuticals provided CGP3466 and RetinaPharma International provided (-)-desmethyldeprenyl.

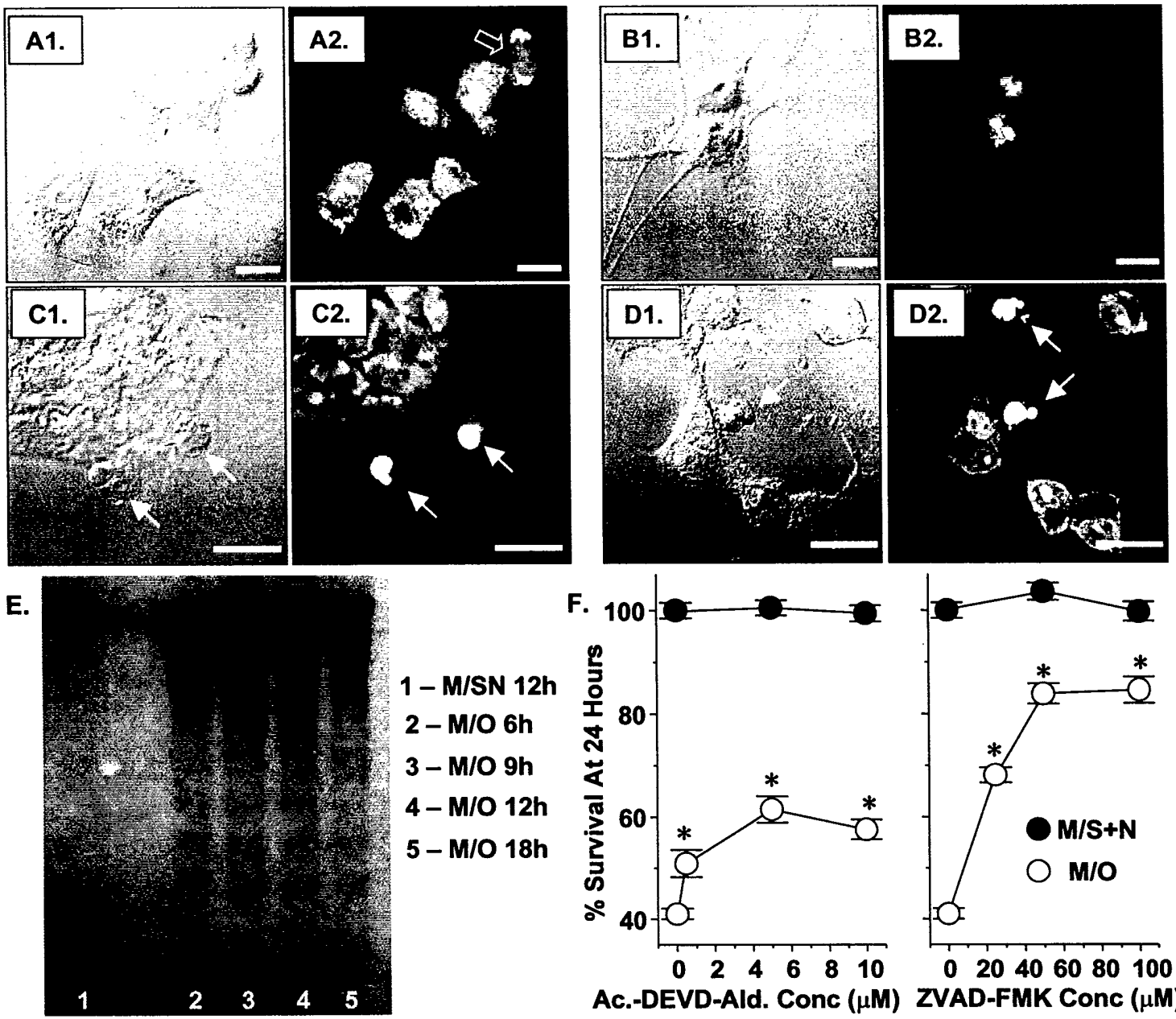


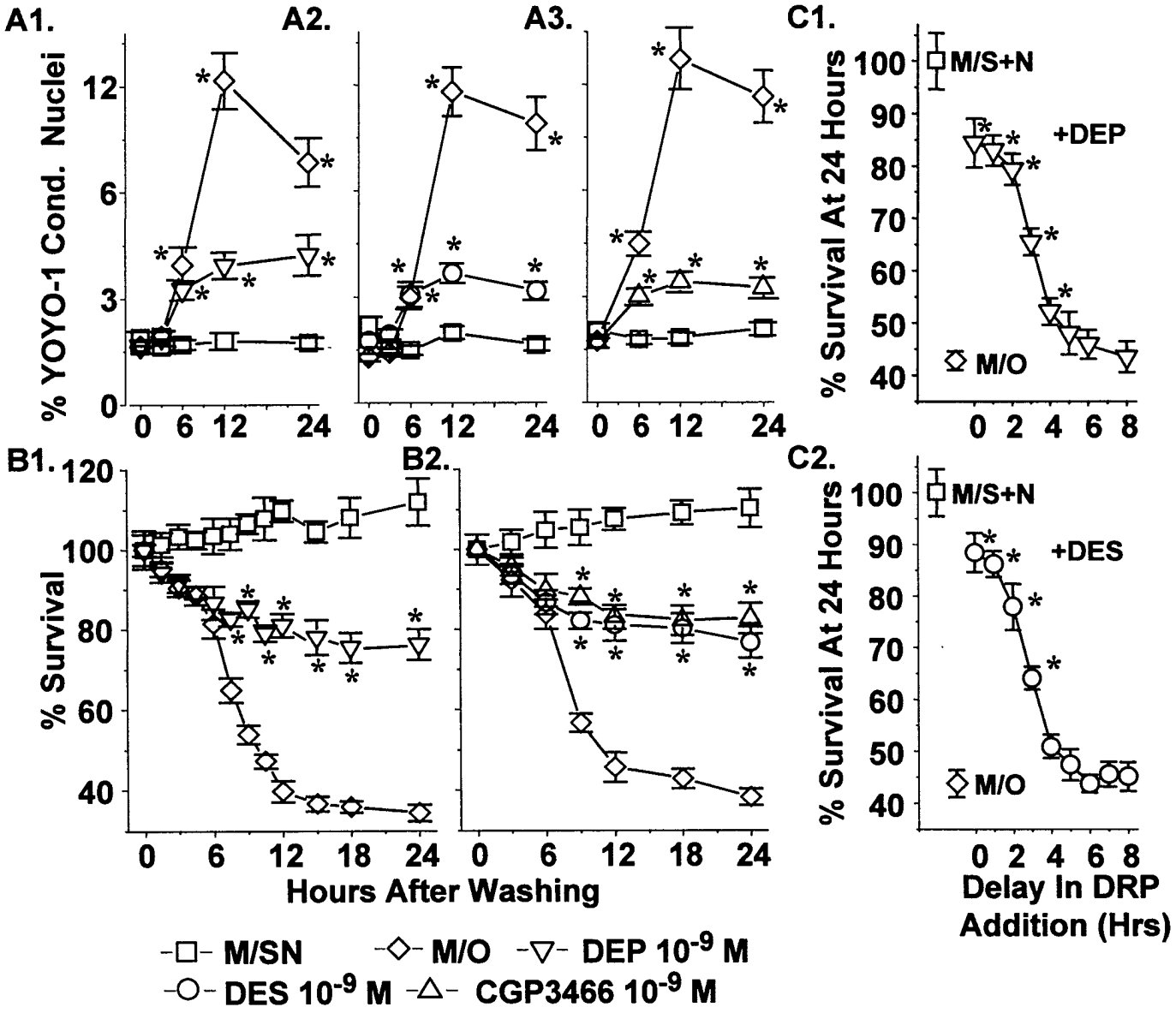
○ Washed and returned to MEM with serum (M/S) or serum plus NGF (M/S+N) – serum or serum and NGF supported

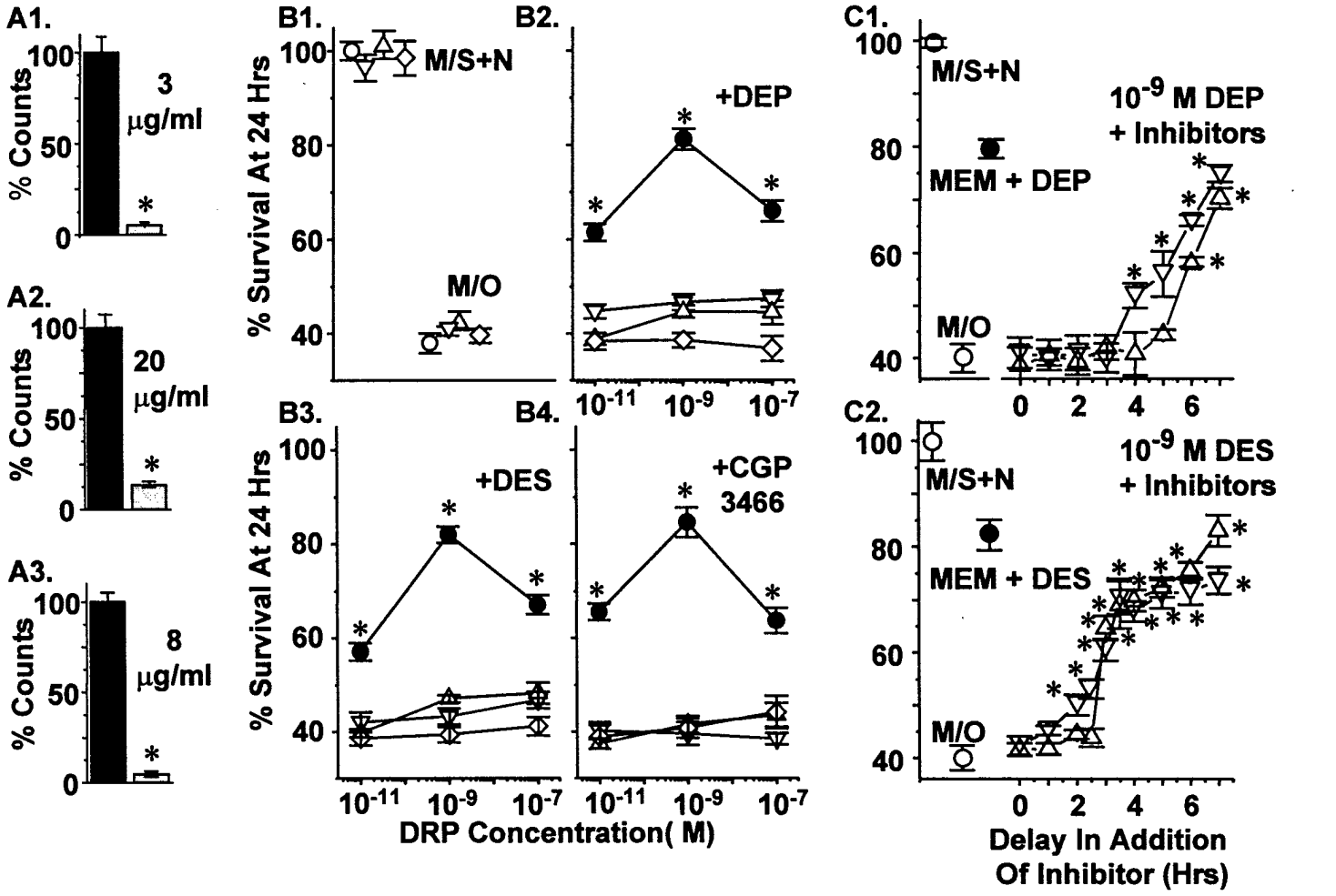
● Washed and placed in MEM only (M/O) – serum or serum and NGF withdrawn

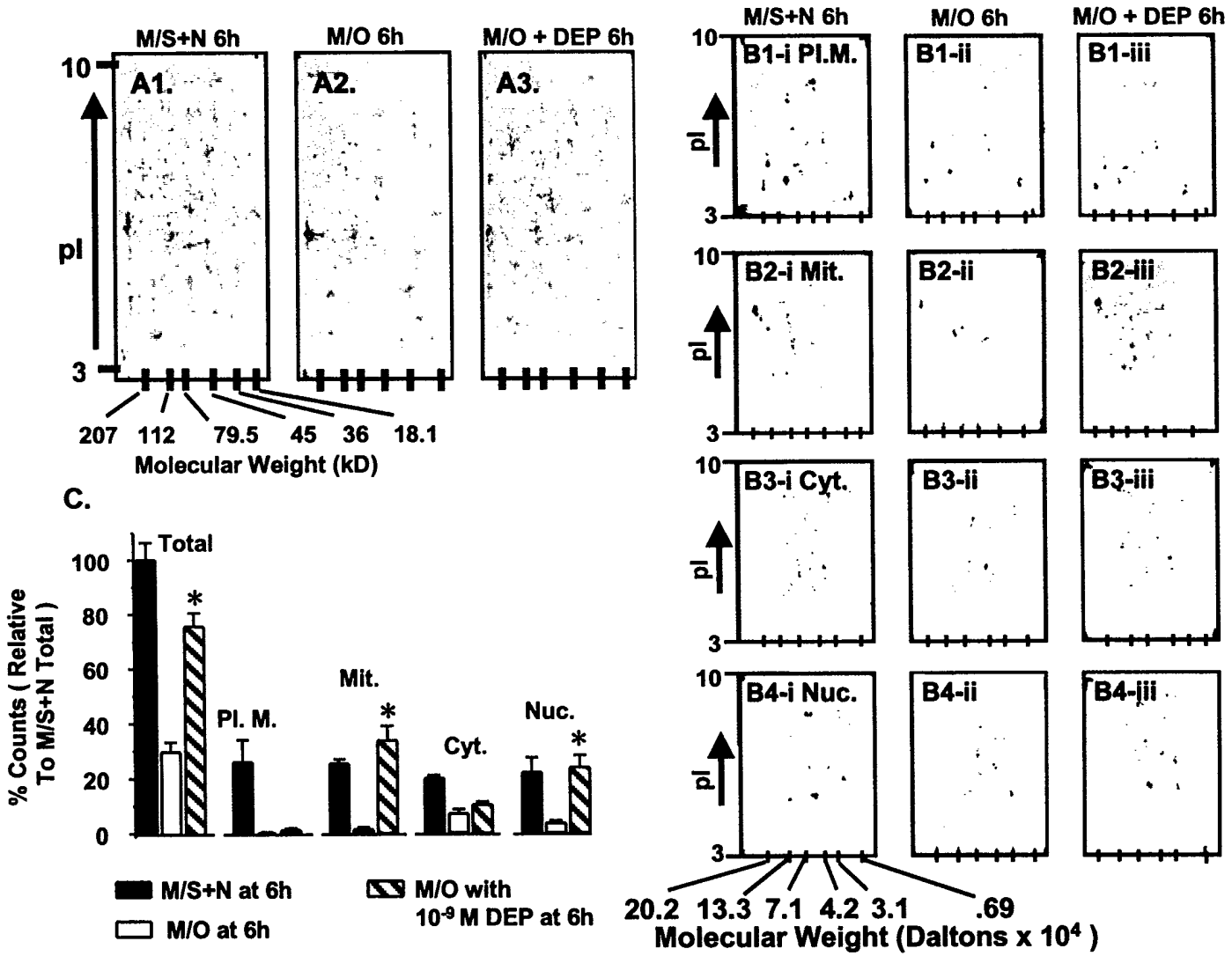
* $p < 0.05$ relative to M/S, M/S+N or M/O

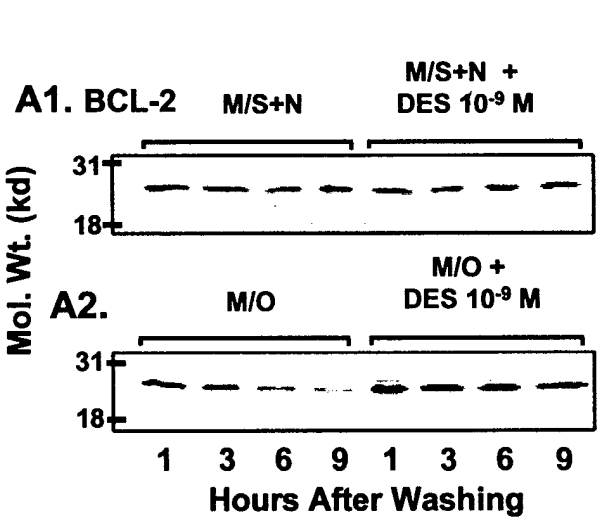

A1.
A2.
A3.
% Survival 24 Hrs After S+N or S Withdrawal
100
80
60
40
-15
-13
-11
-9
-7
-5
DEP Conc.(10^x M)
DES Conc.(10^x M)
CGP3466 Conc.(10^x M)
Day 6 in M/S+N
Day 6 in M/S
Day 6 in M/O
Day 6 in M/O
Day 6 in MEM + DRP
Day 6 in M/S + DRP
B1.
B2.
B3.
% Increase In Survival 24 Hrs After S+N Withdrawal
200
175
150
125
100
0
2
4
6
8
Day Of Addition Of DEP
Day Of Addition Of DES
Day Of Addition Of CGP3466
MEM + DRP
M/O

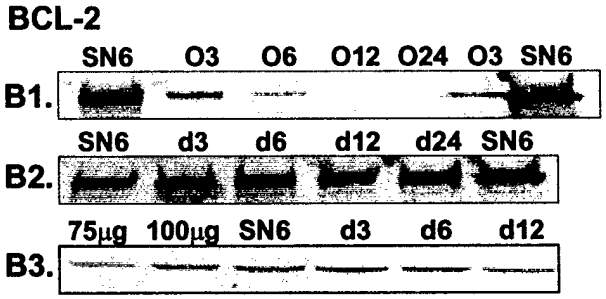

A1.
A2.
B1.
B2.
C1.
C2.
D1.
D2.
E.
1 – M/SN 12h
2 – M/O 6h
3 – M/O 9h
4 – M/O 12h
5 – M/O 18h
1
2
3
4
5
F.
% Survival At 24 Hours
100
80
60
40
0 2 4 6 8 10
Ac.-DEVD-Ald. Conc (μM)
0 20 40 60 80 100
ZVAD-FMK Conc (μM)
M/S+N
M/O

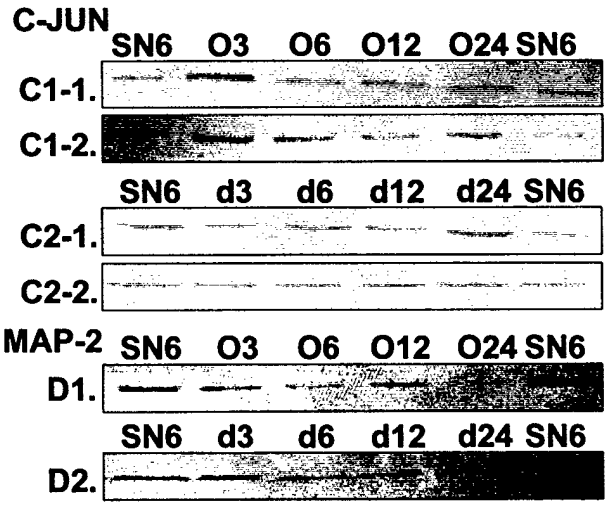

A1.
A2.
A3.
C1.
% YOYO-1 Cond. Nuclei
0
3
6
12
0 6 12 24
M/S+N
+DEP
M/O
% Survival At 24 Hours
100
90
80
70
60
50
40
0 2 4 6 8
B1.
B2.
C2.
% Survival
120
100
80
60
40
0 6 12 18 24
Hours After Washing
+DES
Delay In DRP Addition (Hrs)
M/SN
M/O
DEP 10-9 M
DES 10-9 M
CGP3466 10-9 M

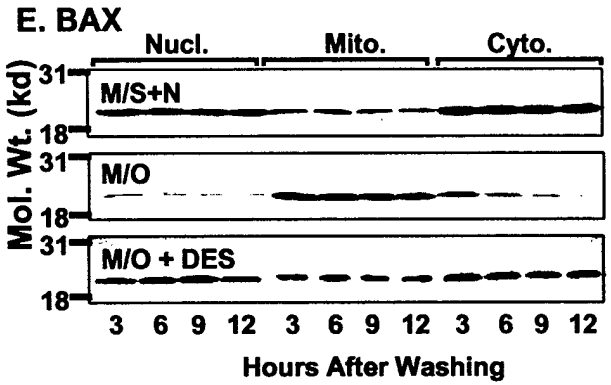

A1.
% Counts
100
50
0
3
µg/ml
A2.
20
µg/ml
A3.
8
µg/ml
B1.
B2.
B3.
B4.
% Survival At 24 Hrs
M/S+N
M/O
+DEP
+DES
+CGP
3466
10-11
10-9
10-7
DRP Concentration(M)
C1.
C2.
10-9 M DEP
+ Inhibitors
MEM + DEP
10-9 M DES
MEM + DES
0
2
4
6
Delay In Addition
Of Inhibitor (Hrs)
-O- No Inhibitor or DRP Addition
-●- DRP Addition
-▽- DRP + Actinomycin D
Addition
-△- DRP + Cycloheximide Addition
-◇- DRP + Camptothecin Addition

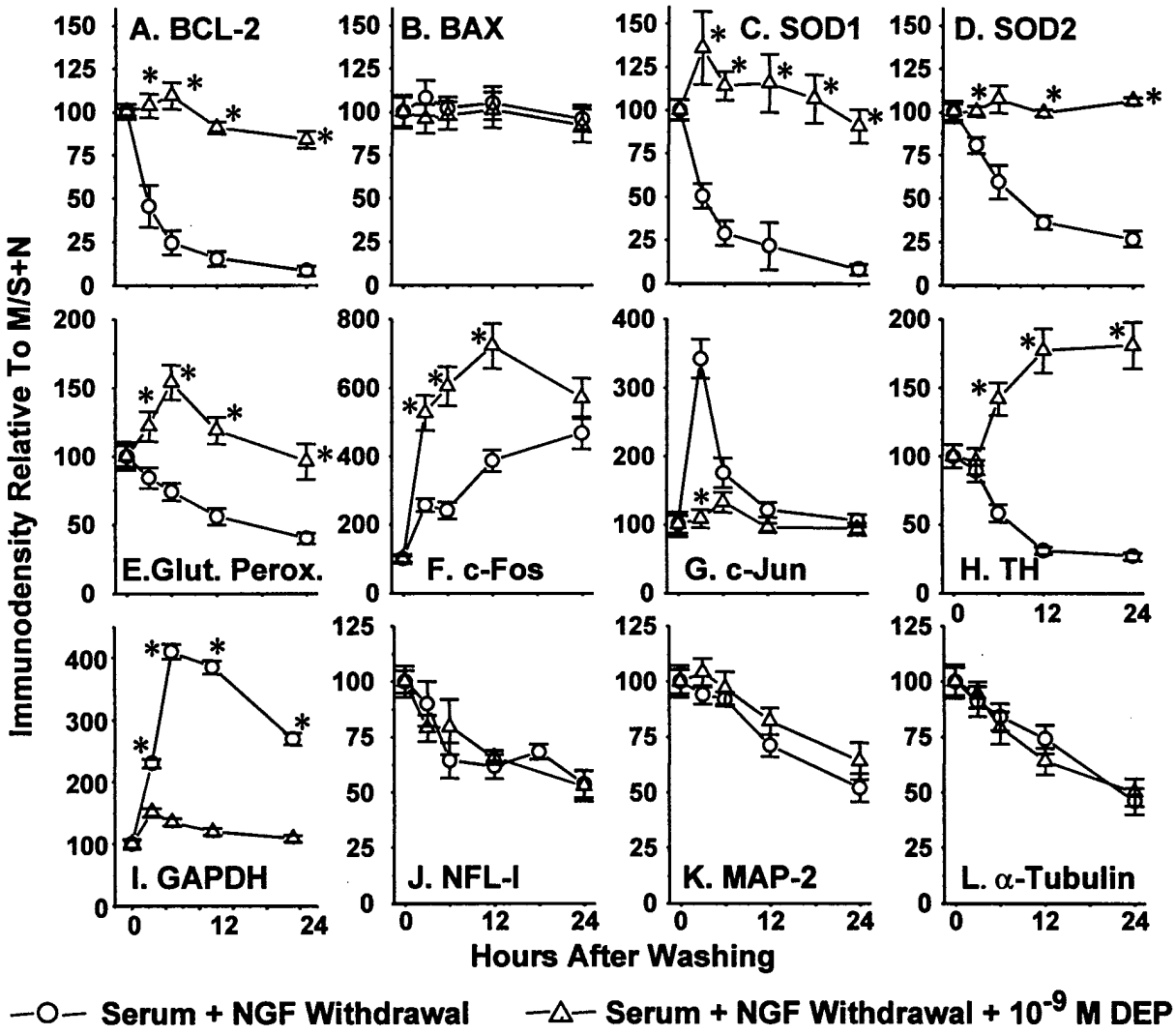

M/S+N 6h
M/O 6h
M/O + DEP 6h
A1.
A2.
A3.
10
3
pI
207 112 79.5 45 36 18.1
Molecular Weight (kD)
B1-i Pl.M.
B1-ii
B1-iii
B2-i Mit.
B2-ii
B2-iii
B3-i Cyt.
B3-ii
B3-iii
B4-i Nuc.
B4-ii
B4-iii
20.2 13.3 7.1 4.2 3.1 .69
Molecular Weight (Daltons x 10⁴)
C.
% Counts (Relative To M/S+N Total)
100
80
60
40
20
0
Total
Pl. M.
Mit.
Cyt.
Nuc.
*
M/S+N at 6h
M/O at 6h
M/O with 10⁻⁹ M DEP at 6h


A1. BCL-2
M/S+N
M/S+N +
DES 10-9 M
31
18
Mol. Wt. (kd)
A2.
M/O
M/O +
DES 10-9 M
31
18
1 3 6 9 1 3 6 9
Hours After Washing


BCL-2
SN6 O3 O6 O12 O24 O3 SN6
B1.
SN6 d3 d6 d12 d24 SN6
B2.
75µg 100µg SN6 d3 d6 d12
B3.


C-JUN
SN6 O3 O6 O12 O24 SN6
C1-1.
C1-2.
SN6 d3 d6 d12 d24 SN6
C2-1.
C2-2.
MAP-2
SN6 O3 O6 O12 O24 SN6
D1.
SN6 d3 d6 d12 d24 SN6
D2.


E. BAX
Nucl.
Mito.
Cyto.
Mol. Wt. (kd)
31
18
M/S+N
M/O
M/O + DES
3 6 9 12 3 6 9 12 3 6 9 12
Hours After Washing


A. BCL-2
B. BAX
C. SOD1
D. SOD2
E.Glut. Perox.
F. c-Fos
G. c-Jun
H. TH
I. GAPDH
J. NFL-I
K. MAP-2
L. α-Tubulin
Immunodensity Relative To M/S+N
Hours After Washing
—O— Serum + NGF Withdrawal
—△— Serum + NGF Withdrawal + 10-9 M DEP