

Differential Susceptibility of Naive and Differentiated PC-12 Cells to Methylglyoxal-Induced Apoptosis: Influence of Cellular Redox

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Abstract: Neuropathologies have been associated with neuronal de-differentiation and oxidative susceptibility. To address whether cellular states determines their oxidative vulnerability, we have challenged naive (undifferentiated) and nerve growth factor-induced differentiated pheochromocytoma (PC12) with methylglyoxal (MG), a model of carbonyl stress. MG dose-dependently induced greater apoptosis (24h) in naive (nPC12) than differentiated (dPC12) cells. This enhanced nPC12 susceptibility was correlated with a high basal oxidized cellular glutathione-to-glutathione disulfide (GSH/GSSG) redox and an MG-induced GSH-to-Disulfide (GSSG plus protein-bound SSG) imbalance. The loss of redox balance occurred at 30 min post-MG exposure, and was prevented by *N*-acetylcysteine (NAC) that was unrelated to *de novo* GSH synthesis. NAC was ineffective when added at 1h post-MG, consistent with an early window of redox signaling. This redox shift was kinetically linked to decreased Bcl-2, increased Bax, and release of mitochondrial cytochrome *c* which preceded caspase-9 and -3 activation and poly ADP-ribose polymerase (PARP) cleavage (1-2h), consistent with mitochondrial apoptotic signaling. The blockade of apoptosis by cyclosporine A supported an involvement of the mitochondrial permeability transition pore. The enhanced vulnerability of nPC12 cells to MG and its relationship to cellular redox shifts will have important implications for understanding differential oxidative vulnerability in various cell types and their transition states.

Key Words: Neurons, GSH/GSSG redox state, protein-SSG, mitochondrial signaling, mitochondrial permeability transition pore, naive and differentiated PC12 cells, methylglyoxal-induced apoptosis.

INTRODUCTION

De-differentiation of neuronal cells, as evidenced by dendritic atrophy and disturbed nerve growth factor-induced neurite extensions, and enhanced oxidative susceptibility have been associated with several neuropathological conditions, such as Alzheimer's disease (AD) and diabetes mellitus (Andersen 2004; Arendt *et al.* 2000a; 2000b; Hamdane *et al.* 2002; Magarinos *et al.* 2000; Reagan *et al.* 1999; Lelkes *et al.* 2001). These findings suggest that the vulnerability of neurons to oxidative challenge and apoptotic death may be linked to a loss of the differentiated phenotype. Indices of reactive oxygen species (ROS) induced damage have been reported within the specific brain region that undergoes selective neurodegeneration (Andersen 2004; Smith *et al.* 1997; Grillo *et al.* 2003), and elevated levels of carbonyl species are observed in the diseased regions of AD and diabetic brain (Markesbery *et al.* 1998; Montine *et al.* 1998; Miyata *et al.* 2003). Given that carbonyl stress has been linked to central nervous system (CNS) complications and dementia progression associated with neurodegenerative states (Picklo *et al.* 2002; Munch *et al.* 2003; Dukic-Stefanovic *et al.* 2001; Miyata *et al.* 2003), the current study was designed to address the vulnerability of a rat pheochromocytoma (PC12) cell line to methylglyoxal (MG),

a precursor of advanced glycated endproducts (AGEs), and a model of carbonyl stress. While susceptibility of cells to oxidant challenge is well established, the responses of cells including neurons, to carbonyl stress are less well characterized. Because MG can augment intracellular oxidative stress due to enhanced production of ROS (Yim *et al.* 1995), it is implicated to play an important role in the pathogenesis of cellular dysfunction related to neurodegenerative complications.

The PC12 cell model was characterized in 1976 (Greene & Tischler 1976) and has since been used extensively to study the cellular and molecular aspects of neuronal apoptosis. A notable characteristic of PC12 cells is that they can readily be induced to differentiate in culture with the neurotrophic factor, nerve growth factor (NGF) whereby cells cease to multiply, assume a neurite-bearing phenotype that resemble mature sympathetic neurons, and exhibit firm attachment to the substratum (Greene & Tischler 1976). Thus, the PC12 cell provides a suitable model to examine the vulnerability of undifferentiated (naive) cells to MG challenge as compared to the responses in differentiated cells, and to determine the mechanisms underlying differential susceptibility of the two cellular transition states.

An important and novel aspect of the current study is the determination of the contribution of cellular glutathione-to-glutathione disulfide (GSH/GSSG) redox to MG-induced apoptosis in PC12 cells given that (a) MG could induce GSH/GSSG imbalance due to its promotion of ROS formation (Yim *et al.* 1995) and (b) our recent results

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implicate GSH-to-GSSG redox shifts as a critical event in the initiation of apoptosis in naive PC12 cells caused by hydroperoxides (Pias & Aw 2002a; 2002b; 2003). Alterations in two key enzymes regulating redox balance, namely GSH peroxidase and GSSG reductase have been reported in AD and diabetic brain (Andersen 2004; Lovell *et al.* 1995; Ulusu *et al.* 2003). Since the mitochondrion is pivotal to the initiation of the apoptotic cascade mediated by oxidative stress and redox imbalance in cells, we further investigated mitochondrial involvement in the MG-induced apoptotic process.

MATERIALS AND METHODS

Materials. Rat adrenal pheochromocytoma (PC-12) cells and F12K medium were obtained from ATCC (Manassas, VA, USA). Mouse nerve growth factor 2.5S (grade II) was obtained from Roche Diagnostics, GmbH (Mannheim, Germany). The following chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA), 4'-diamidino-2-phenylindole (DAPI), DL-buthionine-[S,R] sulfoximine, methyl-glyoxal, N-acetylcysteine, cyclosporin A, 2,4-dinitrofluorobenzene, protease inhibitors (aprotinin, leupeptin, phenylmethylsulfonyl-fluoride, dithiothreitol, glutathione (GSH and GSSG), and iodoacetic acid. Fetal bovine serum and horse serum were from JRH Biosciences (Lenexa, Kansas, USA). Monoclonal antibodies against Bcl-2 (mouse) and Bax (mouse) were from Transduction labs (Lexington, KY, USA); monoclonal antibody against α -actin (mouse) was from Oncogene (Cambridge, MA, USA). Polyclonal antibodies against cytochrome *c* (rabbit), procaspase 3 (CPP32) (goat) and polyADP ribose polymerase (PARP, rabbit) were from Santa Cruz Biologicals (Santa Cruz, CA, USA). Polyclonal antibodies against caspase 8 (mouse) and caspase 9 (mouse) were from Pharmingen (San Diego, CA, USA). Inhibitors of caspase-9 (LEHD-CHO), caspase-8 (IETD-CHO), and caspase-3 (Z-DQMD-FMK) were from Calbiochem (San Diego, CA, USA). Nitrocellulose membranes and Bio-Rad protein dye assay kit were from BIORAD Corporation (Hercules, CA, USA). Hyperfilms and secondary IgG anti-mouse and anti-rabbit antibodies were from Amersham (Arlington Heights, IL, USA). Secondary anti-goat antibody was from Boehringer Mannheim (Indianapolis, IN, USA). Fluorescent mounting media was from DAKO Corporation (Carpinteria, CA, USA). Twelve mm circle number 1 glass cover slips, collagen I-coated 60 mm culture plates, T-25 and T-75 cm₂ flasks were from Discovery Labware (Bedford, MA, USA). All other chemicals were of reagent grade and were purchased from local sources.

Cell Culture and Incubations

Naive PC-12 (nPC12) cells were treated with 75 ng/ml NGF for 1 week to achieve homogenous populations of differentiated PC-12 (dPC12) cells with characteristic neurite outgrowth resembling sympathetic neurons (Greene & Tischler 1976). nPC12 and dPC12 cells were cultured in F12K medium (7 mM glucose with 5 % heat-inactivated fetal bovine serum, 10 % horse serum) on collagen-coated T-25 or T-75 flasks or 60 mm culture plates at 37°C in a 95 % air, 5% CO₂ humidified environment. The culture medium was changed every two days. For all experiments, nPC12

and dPC12 cells were seeded at specified densities the day before the experiment. On the day of the experiment, culture media were replaced with fresh serum-free F12K media. MG was added to cell cultures at final concentrations of 100-1000 μ M. In designated experiments, cells were treated with N-acetylcysteine (NAC), 2 mM; buthionine sulfoximine (BSO), 1 mM; or cyclosporine A, 1 μ M.

Detection of Apoptosis by DAPI Staining

DAPI staining was performed according to Wang *et al.* (1998). PC12 cells (1×10^5) were grown on 12 mm circular glass coverslips in 24-well plates. Cells were treated with 300 μ M MG for 24h, washed with PBS, and fixed with cold 2 % paraformaldehyde for 15 min. After washing with PBS, cells were fixed with cold 70 % ethanol at -20°C for 1 h and stained with 1 μ g/ml DAPI for 30 min in the dark. After two additional PBS washes, slides were mounted using DAKO fluorescent mounting fluid and counted using a fluorescent Olympus B x 50 microscope with a 20x objective. At least six fields of total and apoptotic cells were counted on each slide for a total of 500 cells.

Measurements of GSH and GSSG

Cellular GSH and GSSG were determined by high-performance liquid chromatography (Reed *et al.* 1980). Cells (2×10^6) were cultured in 60 mm culture plates and exposed to 300 μ M MG. In some experiments, cells were pretreated with NAC (2 mM) or NAC plus BSO (1 mM). At times from 0 to 1h, cells were harvested by scraping into ice-cold 5% trichloroacetic acid (TCA). Cell suspensions were centrifuged at 10,000 x rpm (5 min) to remove TCA-insoluble proteins. Acid supernatants were derivatized with 6 mM iodoacetic acid and 1% 2,4-dinitrophenyl fluorobenzene to yield the S-carboxymethyl and 2,4-dinitrophenyl derivatives of GSH and GSSG. Separation of derivatives was performed on a 250 mm x 4.6 mm Alltech Lichrosorb NH₂ 10mm column. GSH/GSSG contents were quantified by comparison to standards derivatized in the same manner. TCA-insoluble proteins were saved for determination of protein-bound disulfides (protein-SSG).

Measurement of Protein-SSG

Protein-SSG was measured by a modified assay of Brigelius *et al.* (1982). TCA-insoluble proteins were solubilized in 0.1 M NaOH and neutralized to pH 8.0 using 1 M HCl. Samples (0.4 ml) were mixed with 0.7 ml phosphate buffer (1.5 mM KH₂PO₄, 6.36 mM K₂HPO₄, 1.57 mM EDTA) containing 2 mM of 5, 5'-dithio-bis-2-nitrobenzoic acid. After 15 min, total absorbance (protein-SH plus protein-SSG) was measured at 410 nm. To a parallel sample, 10 μ l of N-ethylmaleimide was added and incubated for 10 min before absorbance was measured at 410 nm for protein-SSG determination.

Protein Expression of Cytochrome *c*, Bax and Bcl-2 and Activation of Caspases

Lysate preparation. nPC-12 cells (2×10^6) were plated on collagen-coated T-25 flasks and treated with 300 μ M MG for 0 - 6h. Cells were lysed with 500 μ l lysis buffer containing 300 mM NaCl, 50 mM Tris-HCl, 0.5% Triton X-100, 10

$\mu\text{g/ml}$ leupeptin, $10 \mu\text{g/ml}$ aprotinin, 1 mM PMSF, and 1 mM DTT for 30min at 4°C and homogenized. Whole cell extracts were stored at -20°C until Western analyses of Bax, Bcl-2, CPP32, procaspase 8 and 9, and PARP. Cytoplasmic fractions were used for analyses of cytochrome *c* expression.

Western analyses. Equal volumes of 2x sample buffer were added to PC12 cell lysates (20 or $50 \mu\text{g}$ protein). Samples were boiled for 5 min , resolved on 8 or 10% acrylamide gels (100V , 90 min), and transferred to nitrocellulose membranes. The membranes were probed individually with anti-Bax, Bcl-2, cytochrome *c*, CPP32, procaspase 9, procaspase 8, or PARP ($1:250$ - $1:1000$). The secondary antibody corresponded to the respective primary antibodies (mouse, rabbit or goat) and was conjugated to horseradish peroxidase ($1:1000$). Detection of chemiluminescence was performed with an ECL Western blotting detection reagent according to manufacturer's recommendation. Each membrane was stripped (6.25 mM Tris pH 6.7 , 2% SDS, 100 mM mercaptoethanol) and probed for β -actin to verify equal protein loading.

Protein Assay. Protein was measured using the Bio-Rad Protein Assay kit according to manufacturer's protocol.

Statistical Analysis. Results are expressed as mean \pm SEM. Data were analyzed using a one-way ANOVA with Fisher corrections for multiple comparisons. $P < 0.05$ were considered statistically significant.

RESULTS

MG Induces Differential Apoptosis in nPC12 and dPC12 Cells

A 24h culture of control PC12 cells, regardless of cellular state, exhibited $\sim 5\%$ cell death that was characteristic of apoptosis and consistent with normal cell turnover. MG dose-dependently induced greater apoptosis at 24h in nPC12 than in dPC12 cells over the range of 100 - $1000 \mu\text{M}$ MG (Fig. 1). At $300 \mu\text{M}$ MG, apoptosis in nPC-12 and dPC12 cells was respectively, $19.4 \pm 0.9\%$ and $11.6 \pm 0.4\%$. Total cell number at 24h for the two cellular states were not different at this MG dose (data not shown), indicating a lack of necrosis. In subsequent experiments, $300 \mu\text{M}$ MG was used to examine the relationship between MG-induced PC-12 apoptosis and cellular redox and apoptotic signaling.

MG Induces Differential Changes in Cellular Redox in nPC12 and dPC12 Cells

To determine a role for cellular redox in PC12 apoptosis, we quantified baseline GSH/GSSG and those after MG challenge. Fig. 2 shows that basal GSH-to-GSSG ratio in nPC12 cells was significantly lower than that in dPC12 cells, due primarily to elevated GSSG levels, consistent with a higher baseline oxidative stress in nPC-12 cells. Initial time course experiments (0 - 1h) showed that $300 \mu\text{M}$ MG induces GSH oxidation maximally at 0.5h post-MG treatment (data not shown), indicating an early and rapid induction of redox imbalance. At 0.5h , MG induced a larger GSH decrease in nPC12 cells (Fig. 2A). Interestingly, GSH loss was significantly associated with GSSG decrease but protein-SSG increase in nPC12 and dPC12 cells (Fig. 2B & 2C),

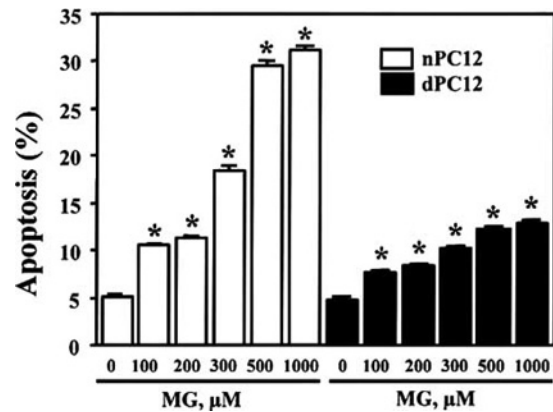


Fig. (1). Differential susceptibility of nPC12 and dPC12 cells to methylglyoxal (MG). PC12 cells were incubated without or with MG (100 to $1000 \mu\text{M}$), and apoptosis was determined at 24 h . Values are means \pm SEM for $n = 5$ preparations. $*p < 0.05$ vs corresponding controls (without MG).

suggesting participation of soluble and protein bound thiol/disulfide pools in redox homeostasis. Consequently, the indicator of cellular redox state was best expressed as the change in GSH- to-combined Disulfide (GSSG + protein-SSG) ratio. Fig. 2D shows that MG induced a greater decrease in the GSH-to-combined Disulfide ratio in nPC-12 than in dPC12 cells. The total glutathione pool (soluble plus protein-bound thiol/disulfide) was unaltered (data not shown), indicating that MG elicited redistribution, rather than loss, of the cellular thiol-disulfide pools. Collectively, these results are consistent with elevated oxidative stress in MG-treated nPC12 cells which contributed to their enhanced vulnerability.

MG-Induced PC-12 Cell Apoptosis is Abrogated by Exogenous NAC When Administered Pre-MG but not Post-MG Treatment

To confirm the contribution of cellular redox, PC12 cells were treated with the thiol antioxidant, NAC at 0.5h before or at 1 h after exposure to $300 \mu\text{M}$ MG. The results show that pre-treatment with NAC significantly attenuated MG-induced apoptosis in both nPC12 and dPC12 cells (Fig. 3). Notably, the addition of NAC at 1 h post-MG treatment and at a time after induction of redox imbalance did not confer cytoprotection against MG challenge. Taken together, these results indicate that cellular redox is a mediator of MG-induced PC12 apoptosis and that the loss of redox balance at 30 min after drug treatment constitutes an early signaling event in apoptosis initiation.

MG-Induced Redox Imbalance is Prevented by NAC that is Unrelated to *de Novo* GSH Synthesis

The results in Fig. 4 show that cellular redox homeostasis was restored in the presence of NAC. In nPC12 cells, NAC pretreatment resulted in significant attenuation of GSSG and protein-SSG (Fig. 4B & C), thereby preserving the cellular soluble and protein-bound thiol pools and the GSH-to-combined Disulfide ratio (Fig. 4D). Interestingly, in dPC12 cells, NAC elevated GSH to levels significantly above, and

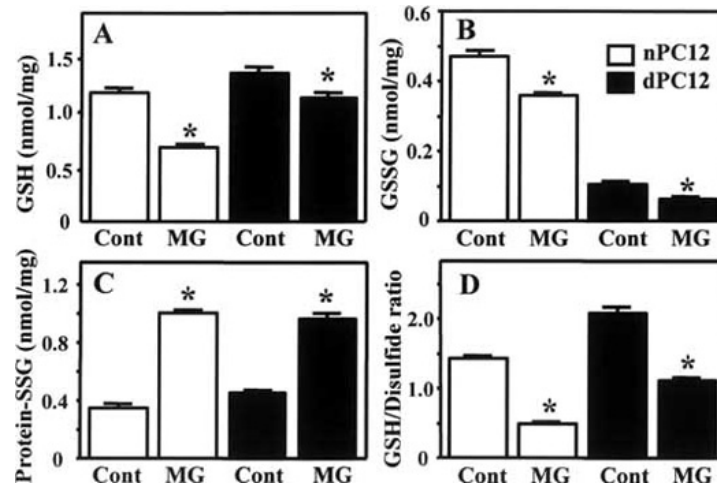


Fig. (2). MG-induced changes in intracellular redox in nPC12 and dPC12 cells. PC12 cells were incubated without or with 300 μ M MG for 0.5 h and GSH/GSSG and protein-SG were determined. A, GSH; B, GSSG; C, protein-SG; D, GSH-to-Disulfide ratio. The disulfide pool comprises acid-soluble GSSG plus protein-SG. Values are means \pm SEM for n = 9 preparations. * p <0.05 vs corresponding controls (without MG).

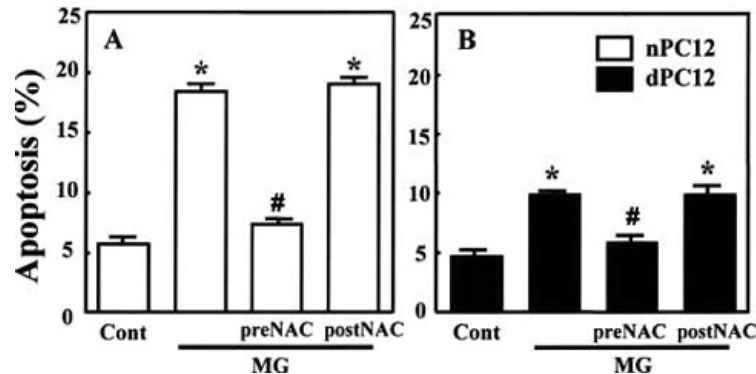


Fig. (3). Effect of NAC on MG-induced apoptosis in nPC12 (A) and dPC12 (B) cells. PC12 cells were treated with 2 mM NAC at 0.5 h before (preNAC) or at 1 h after (postNAC) exposure to 300 μ M MG. Apoptosis was determined at 24 h. Values are means \pm SEM for n = 5 preparations. * p <0.05 vs corresponding controls (without MG); # p <0.05 vs corresponding MG-treated.

protein-SG to values significantly below, control values (Fig. 4A & C). This resulted in a substantially higher GSH-to- combined Disulfide ratio (Fig. 4D), consistent with the achievement of a highly reduced redox state in dPC12 cells. BSO addition to NAC-treated nPC12 and dPC12 cells was without effect, indicating that the restoration of cellular GSH by NAC was unrelated to its role as a precursor in *de novo* GSH synthesis.

Mitochondrial Involvement in PC12 Apoptosis and Expression Kinetics of Central Protein Components of the Mitochondrial Apoptotic Pathway

The above results underscore the importance of the cellular GSH/GSSG redox in determining the vulnerability of nPC12 cells to MG challenge. We recently found that a loss of GSH-to-GSSG balance mediated mitochondrial apoptotic signaling in hydroperoxide- induced nPC12 apoptosis (Pias & Aw 2002a; 2002b; 2003). To evaluate

mitochondrial involvement in MG-mediated cell apoptosis, nPC12 cells were treated with inhibitors of caspases-3, -8, or -9, the latter two being initiator caspases of the death receptor or mitochondrial pathways, respectively (Thornberry *et al.* 1998). The results show that nPC12 apoptosis was significantly attenuated by inhibition of caspase-3 or -9, while treatment with caspase-8 inhibitor was without effect (Fig. 5), indicating that MG mediated nPC-12 apoptosis by direct mitochondria signals. The kinetics of mitochondrial apoptotic signaling was examined by expressions of the central components of this pathway, namely cytochrome *c*, Bax and Bcl-2 (Yang *et al.* 1997). Cytosolic cytochrome *c* expression was absent in control nPC12 cells (Fig. 6A), but increased significantly at 0.5h after MG treatment, indicating its release from the mitochondria. Expression remained elevated for 2h and returned to baseline levels by 4 - 6h. Because mitochondrial cytochrome *c* release has been linked to the function of anti-

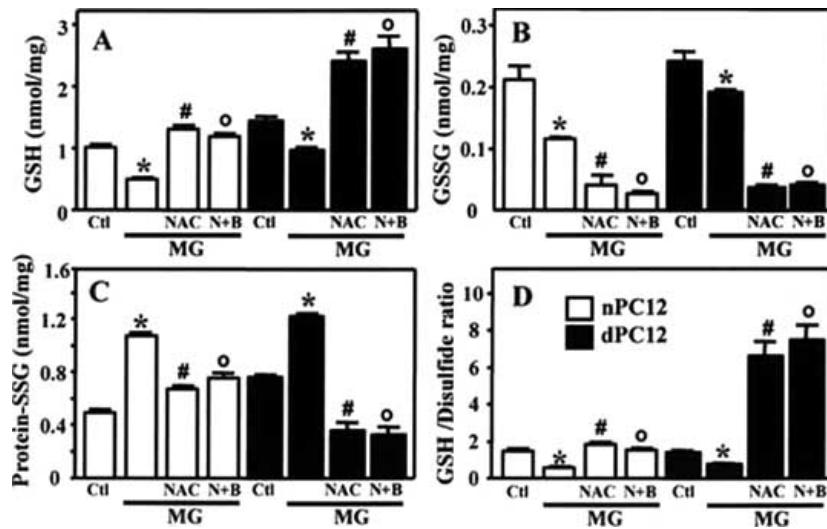


Fig. (4). Effect of NAC and BSO on MG-induced intracellular redox changes in nPC12 and dPC12 cells. PC12 cells were treated with 2 mM NAC or NAC plus 1 mM BSO (N+B) for 0.5 h prior to 300 μ M MG exposure. Cellular GSH/GSSG and protein-SSG were determined at 0.5 h. A, GSH; B, GSSG; C, protein-SSG; D, GSH-to-Disulfide ratio. The disulfide pool comprises acid-soluble GSSG plus protein-SSG. Values are means \pm SEM for n = 9 preparations. * p <0.05 vs corresponding controls (without MG); # p <0.05 vs corresponding MG-treated; °not significant vs NAC.

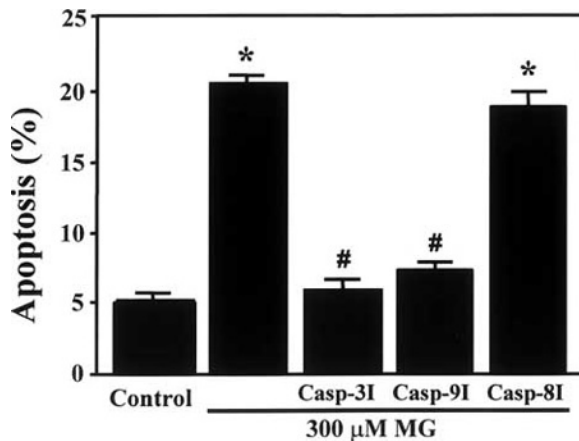


Fig. (5). Attenuation of MG-induced nPC-12 apoptosis by inhibitors of caspase-3 and -9, but not caspase-8. nPC12 cells were preincubated for 0.5 h without or with inhibitors of caspase-3 (1 μ M), caspase-9 (10 μ M) or caspase-8 (10 μ M) prior to 300 μ M MG treatment. Apoptosis was determined at 24 h. Values are means \pm SEM for n = 5 preparations. * p <0.05 vs control (without MG); # p <0.05 vs MG-treated.

apoptotic Bcl-2 and pro-apoptotic Bax (Yang *et al.* 1997), levels of these mitochondrial proteins were determined. The results show that baseline expression of Bcl-2 was high and decreased from 0.5h to 6h post MG exposure (Fig. 6B). In comparison, Bax increased transiently at 0.5h and decreased at 1h to below baseline levels for 6h (Fig. 6B). Notably, the significant loss in Bcl-2 expression relative to increase in Bax expression at 0.5h corresponded kinetically to the induction of redox imbalance (Figs. 2 & 4) and appearance of cytosolic cytochrome *c* (Fig. 6A) at this time, consistent

with an early redox-mediated shift from an anti-apoptotic to a pro-apoptotic phenotype.

MG-Induced Caspase-9 and -3 Activation and PARP Cleavage Kinetically Correlated with Loss of Mitochondrial Integrity

Activation of caspases was examined as downstream events from mitochondrial signaling. Pro-caspase-9 level was high at pre- and at 0.5h post-MG treatment (Fig. 7A); thereafter the levels decreased markedly at 1h, consistent with cleavage of the pro-enzyme to active caspase-9. Caspase-3 activation was evaluated by cleavage of pro-caspase-3 (CPP-32) and its target substrate, PARP. In agreement with previous findings, baseline CPP32 expression in nPC12 cells was low (Pias & Aw 2002a; 2002b; 2003). MG caused a marked increase in CPP32 levels at 1h followed by significant decreases at 2 – 6h (Fig. 7A), indicating an initial upregulation of pro-caspase-3 and its subsequent activation. Control cells expressed one prominent protein band that correlated to naive PARP (116 kDa; Fig. 7B). Significant PARP cleavage to its 85 kDa product occurred at 2 - 6h (Fig. 7B), a time frame that was preceded by caspase-3 activation at 1h (Fig. 7A). Consistent with a lack of a role for the death receptor pathway, caspase-8 expression levels and activation status were unchanged at all time points (Fig. 7A).

Mitochondrial Permeability Transition Pore Mediates nPC12 Apoptosis

Since mitochondrial permeability transition (MPT) has been implicated in some modes of apoptotic cell death to facilitate the release of cytochrome *c* (Walter *et al.* 1998), we determined the contribution of MPT to MG-induced apoptosis of nPC12 cells. Fig. 8 shows that pretreatment of

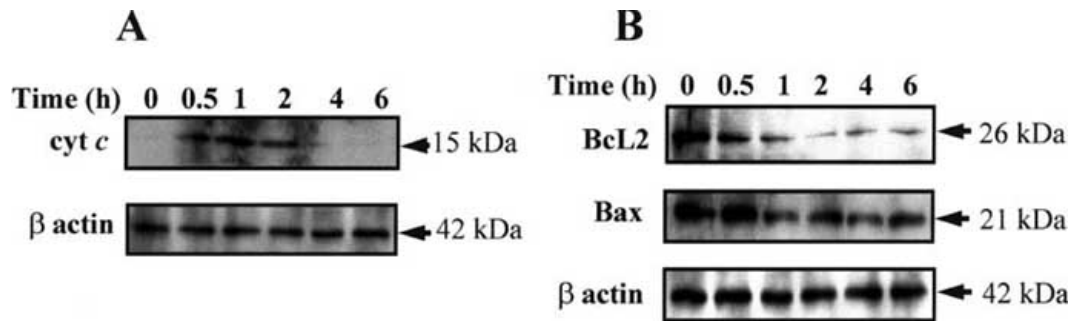


Fig. (6): MG-induced mitochondrial apoptotic signaling: Protein expression of cytochrome *c*, Bax, and Bcl-2. nPC12 cells were treated with 300 μ M MG and Western analyses of cytochrome *c* or Bax and Bcl-2 were performed on cytosolic or total cell lysates, respectively. Results shown are one representative of three immunoblots for each protein. β -actin verified equal protein loading.

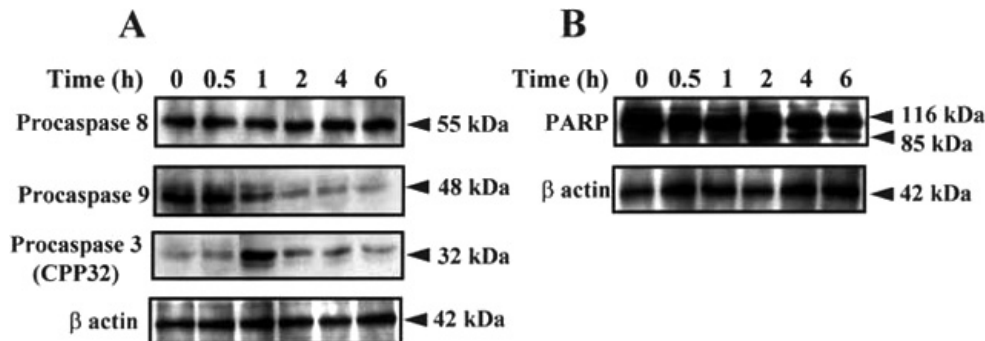


Fig. (7). MG-induced caspase-9 and -3 activation and PARP cleavage. nPC12 cells were treated with 300 μ M MG and Western analysis of caspase activation and PARP cleavage were performed on total cell lysates. Results shown are one representative of three immunoblots for caspase-9, -8 and -3 (CPP32), and PARP. β -actin verified equal protein loading.

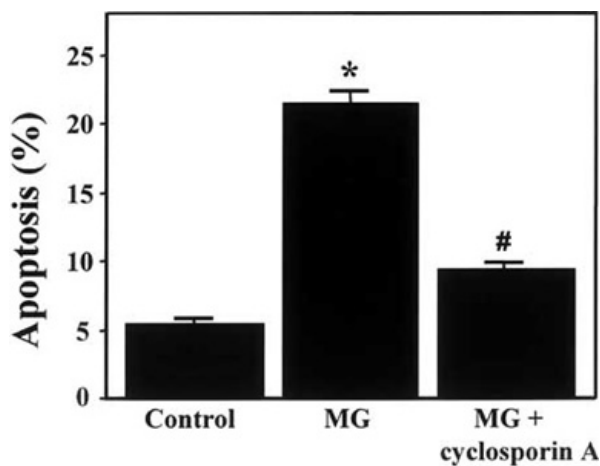


Fig. (8). MG-induced nPC-12 apoptosis is inhibited by cyclosporine A. nPC-12 cells were incubated without or with 1 μ M cyclosporine A prior to 300 μ M MG exposure. Apoptosis was determined at 24 h. Values are means \pm SEM for $n = 9$ preparations. * $p < 0.05$ vs control (without MG); # $p < 0.05$ vs MG-treated.

nPC12 cells with cyclosporine A, an inhibitor of pore function, significantly attenuated MG-induced apoptosis, confirming a role for MPT in mediating apoptosis during MG challenge.

DISCUSSION

The current study demonstrated that the susceptibility of PC12 cells to MG is a function of the cellular transition state in that differentiated cells are more resistant than naive, undifferentiated cells. Our finding is in agreement with previous studies that diabetic rat hippocampal neurons accompanying morphological changes reminiscent of a poorly differentiated state were susceptible to MG exposure (Di Loreto *et al.* 2004). Three lines of evidence support our conclusion that cellular redox (GSH/GSSG and protein-bound thiol/disulfides) is a key determinant of nPC12 vulnerability. First, enhanced nPC12 apoptosis was correlated with a higher basal oxidative stress as evidenced by decreased GSH-to-GSSG ratio despite similar total glutathione pools in nPC12 and dPC12 cells. The higher oxidative stress in nPC12 cells is likely a result of lower basal activities of the GSH redox enzymes (GSH peroxidase, GSSG reductase, and glucose 6-phosphate dehydrogenase) (Ekshyyan and Aw, unpublished results). Second, the degree of redox imbalance (decrease in GSH with respect to the combined disulfides of GSSG and protein-SSG) caused by MG was greater in nPC12 than dPC12 cells. Finally, NAC pretreatment resulted in greater cellular GSH recovery, protein-SSG reduction, and 4-fold higher GSH-to-combined Disulfide ratio in dPC12 than nPC12 cells. Collectively, these findings suggest a differential sensitivity of redox control in nPC12 and dPC12 cells which likely contributed

to their differential vulnerability to MG challenge. This conclusion is supported by previous observations that the differential susceptibility to oxidant stress among various cell types was closely linked to the extent of GSH/GSSG redox imbalance (Pias & Aw 2003; Amicarelli *et al.* 2003).

That improved redox status directly correlated with attenuated MG-induced apoptosis supports a role for redox in modulation of apoptosis (Pias & Aw 2002a; 2002b; 2003). We found that the induction of apoptosis, regardless of naive or differentiated status, was mediated by an early (30 min) MG-induced loss of redox balance which was effectively prevented by NAC pretreatment. The failure of NAC to protect against apoptotic death when added post-MG at a time after the induction of redox shifts (at 1h) suggests that this initial disruption of redox status is a critical upstream event in mediating PC12 cell apoptosis. Notably, cytoprotection can only be achieved with blockade of redox disruption within this early time window of 30 min; once redox imbalance is initiated, the administration of the thiol antioxidant was ineffective in preventing the progression of PC12 apoptosis to its biological endpoint at 24h. These results support a causal link between cellular redox and apoptotic signaling. Previous studies from our laboratory and others have similarly demonstrated the importance of an early induction of redox imbalance (15 min - 1h) in oxidant mediated apoptosis in different cell types (Pias & Aw 2002a; 2002b; 2003; Wang & Aw 2000; Mirkovic *et al.* 1997). Mechanistically, NAC could serve as a cysteine precursor for *de novo* GSH synthesis. However, the failure of BSO to block the effect of NAC indicates that GSH synthesis was not a major mechanism. Rather, NAC could function as a reductant in MG elimination, thereby attenuating GSH oxidation and sparing the cellular GSH and protein-SH pools.

A notable observation in the current study is the involvement of soluble and protein-bound thiol and disulfide pools in MG-mediated PC12 apoptosis. It is interesting that GSH loss following MG treatment did not result in an expected increase in GSSG that is typical during oxidative challenge, but rather in significant formation of protein-SSG. The lack of GSSG increase may be accounted for by its rapid interaction with accessible cysteinyl thiols of cellular proteins to form protein-SSG (Rossi *et al.* 1995; Rebrin *et al.* 2003; Collison *et al.* 1987, Fig. 9) via a thioltransferase catalyzed reaction (Burchill *et al.* 1978) given the existence of an equilibrium between free GSSG and protein-SH. The increase in protein-SSG could also be the result of oxidation of protein sulfhydryls (Fig. 9) consequent to protein-carbonyl compound crosslinks, a potential mechanism in intracellular signaling (Akhand *et al.* 1999; Akhand *et al.* 2001). This shift towards protein-SSG formation is consistent with a pro-oxidizing shift (Rossi *et al.* 1995; Rebrin *et al.* 2003; Burchill *et al.* 1978); indeed previous studies have demonstrated increases in protein-SSG concentrations after oxidative stress (Rossi *et al.* 1995). It is notable that, in contrast to MG challenge, only the soluble pool of GSH/GSSG was involved in nPC12 apoptosis regulation after *tert*-butylhydroperoxide challenge (Pias & Aw 2002a; 2003). Clearly, the differential involvement of the different thiol/disulfide compartments during MG- or hydroperoxide challenge is oxidant specific. Furthermore,

different steady state concentrations of GSSG achievable by these two oxidants may differentially influence the thioltransferase-catalyzed reaction with protein cysteine moieties.

The mechanism by which MG-induced redox shifts mediates PC-12 cell apoptosis is unresolved. One mechanism may involve target-specific thiol oxidation of the redox-sensitive MPT to regulate pore opening and closure. GSH has been shown to interact with MPT to control the threshold potential for pore opening, and this event is closely associated with the function of Bax and Bcl-2 in regulating cytochrome *c* translocation from the mitochondria to the cytosol (Halestrap *et al.* 1997; Costantini *et al.* 1996). Previous studies have demonstrated that thiol oxidation in the MPT pore through formation of disulfide crosslinks blocked Bcl-2 function (Marchetti *et al.* 1997; Zamzami *et al.* 1998). Our current finding that MPT is involved in MG-mediated apoptosis and that MG significantly induced oxidation of protein-SH may lend itself to such a regulation. In other studies, involvement of the p38 mitogen activated protein (MAP) kinase and c-Jun N-terminal kinase (JNK) in MG-induced apoptotic cell death has been reported (Liu *et al.* 2003; Du *et al.* 2000), but a role for redox in modulating the MAP kinase cascade has not been demonstrated. Ongoing efforts in our laboratory are devoted to this area of investigation.

The sequelae of events in MG-mediated nPC12 apoptosis are consistent with mitochondrial initiated apoptotic signaling (Cai *et al.* 1998). The time courses of loss of redox balance, altered Bax and Bcl-2 expression, release of mitochondrial cytochrome *c*, activation of caspase-9 and -3, and cleavage of PARP is the first reported kinetic sequence of mitochondrial signaling events for MG-induced apoptosis. The mitochondrial protein expression and caspase activation kinetics (30 min - 2h) are consistent with these events being downstream of the induction of redox imbalance (30 min) and upstream of apoptotic death (24h), thus supporting the paradigm that redox activation of mitochondrial signaling determines apoptosis progression during MG challenge, similar to other oxidants (Pias & Aw 2002a; 2003; Cai *et al.* 1998; Green *et al.* 1998; Cai *et al.* 2002). Previous studies have implicated dysfunctional brain mitochondria in diabetic tissue vulnerability to oxidative stress (Santos *et al.* 2001; Raza *et al.* 2004), and that mitochondrial signaling elicits Bax increases, cytochrome *c* translocation, and caspases activation and PARP cleavage in target organs (Li *et al.* 2002; Cai *et al.* 2002; Piotrowski *et al.* 2001; Federici *et al.* 2001; Skaper 2003). The findings of decreased Bcl-2 concomitant with increased Bax expression in neurons with neurofibrillary tangles (MacGibbon *et al.* 1997; Vyas *et al.* 1997), and the presence of activated caspase-3 within autophagic granules (Selznick *et al.* 1999) are evidence of apoptotic signaling in AD. Neurotoxicity from amyloid- $(A\beta)$ peptides and other carbonyl-terminal fragments (CTFs) of the amyloid- β protein precursor (A β PP) (Haass *et al.* 1992; Hartmann *et al.* 1997) is a key event in AD. Woltjer *et al.* (2003) recently demonstrated that A β CTF cytotoxicity was enhanced by MG in a human neuroblastoma cell line that conditionally expresses CTFs of the A β PP. Other studies showed that MG induced neurotoxicity of cultured cortical and hippocampal neurons (Kikuchi *et al.* 1999; Di Loreto *et*

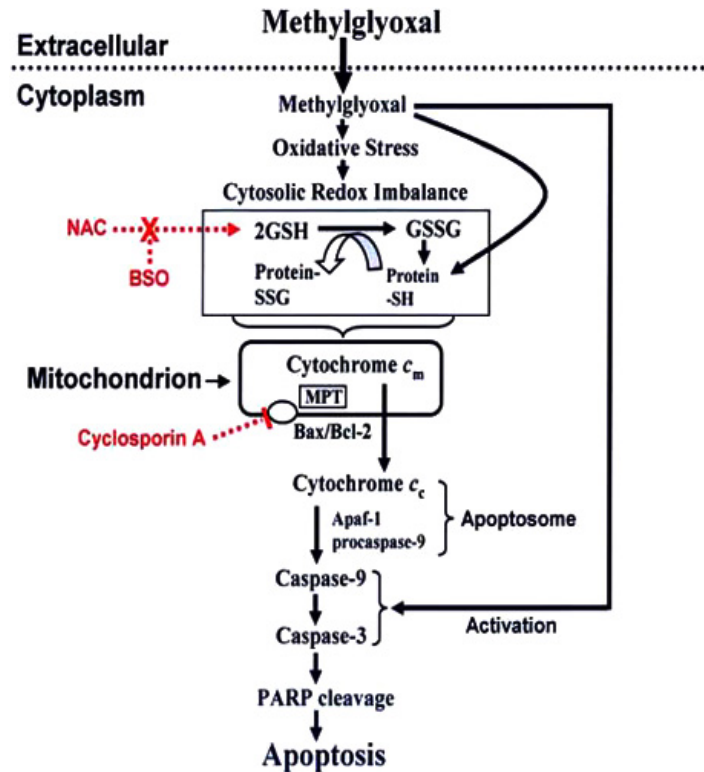


Fig. (9). Working model summarizing MG-induced redox shifts and the pathway leading to PC12 apoptosis. The model proposes that methylglyoxal induces cellular redox imbalance through generalized oxidative stress and/or oxidation of protein thiols which causes mitochondrial dysfunction, including permeability transition that initiates the apoptotic cascade involving mitochondrial cytochrome *c* release and death caspase activation. Literature evidence suggests that in certain cell types, MG can directly activate caspases *via* protein-carbonyl compound crosslinks (Akland *et al.* 2001). Our data, however, indicate that the differential susceptibility of nPC12 and dPC12 cells to MG challenge lies at the level of control of cellular GSH/Disulfide status preceding the initiation of apoptotic signaling. In this regard, the relative resistance of dPC12 cells is attributed to their ability to better maintain the cellular GSH/Disulfide status. Dotted red lines indicate the location of substrate (NAC) entry and inhibitor (BSO, cyclosporine A) action.

al. 2004), a process that could contribute to AD progression. Indices of ROS damage, and markers of lipid peroxidation, such as 4-hydroxynoneal (HNE) and acrolein, have been identified in the cortex and hippocampus of patients with AD (Butterfield *et al.* 2002; Andersen 2004), consistent with an involvement of reactive oxygen species (ROS) (Andersen 2004). Carbonyl compounds like HNE, acrolein and MG that enhance ROS production are therefore, important mediators of neuronal death in AD pathogenesis (Picklo *et al.* 2002). Our current study suggests that MG-induced redox imbalance could be a major contributor to neuronal loss through enhanced cell apoptosis in disease progression.

The 300 μM concentration of MG used in this study was similar those used by other investigators in *in vitro* studies. Previous studies have demonstrated that only $\sim 1.8\%$ of exogenous MG is taken up into cells (Che *et al.* 1997); thus at 300 μM exogenous MG, a final MG level of 5.4 μM will be achieved intracellularly which is comparable to the plasma concentrations of 5-10 μM in diabetic patients (Beisswenger *et al.* 1999; Liu *et al.* 2003; Phillips *et al.* 1993). Additionally, a 6-fold higher than plasma concentrations can be attained in the diabetic tissues like the

kidney (Phillips *et al.* 1993). Although MG concentrations in the CNS in AD and diabetes are not known, elevated levels of various carbonyl compounds in the diseased regions of AD and diabetic brains have been demonstrated (Markesbery *et al.* 1998; Montine *et al.* 1998; Miyata *et al.* 2003).

In summary, we have demonstrated a differential susceptibility of naive and differentiated PC12 cells to MG. The enhanced apoptosis of naive cells corresponded to a higher basal oxidative stress and a decreased capacity to maintain a highly reduced redox state during MG challenge. Our study is the first report of a direct link between cellular redox and the induction of mitochondrial apoptotic signaling induced by carbonyl stress. The data show that MG-induced redox perturbation is causally linked to mitochondrial apoptotic signaling involving proapoptotic shifts in Bax/Bcl2 expression, mitochondrial cytochrome *c* translocation, and caspases-9 and -3 activation. A working model could be as summarized in Fig. 9: methylglyoxal induces cellular redox imbalance through generalized oxidative stress and/or oxidation of protein thiols which subsequently causes mitochondrial dysfunction, including permeability transition that initiates the apoptotic cascade *via*

mitochondrial release of cytochrome *c* and activation of death caspases leading to cell apoptosis. Given that neurons in degenerative diseases such as AD and diabetes, exhibit cell morphology reminiscent of a poorly differentiated state, the enhanced vulnerability of nPC12 cells to MG and its relationship to cellular redox shifts will have important implications for understanding oxidative challenge in CNS complications and neuronal dysfunction associated with these neuropathological processes.

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