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The Effect of Endogenous Dopamine in Rotenone-Induced Toxicity in PC12 Cells

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ABSTRACT

Deficiencies in Complex I have been observed in Parkinson's disease (PD) patients. Systemic exposure to rotenone, a Complex I inhibitor, has been shown to lead to selective dopaminergic cell death *in vivo* and toxicity in many *in vitro* models, including dopaminergic cell cultures. However, it remains unclear why rotenone seems to affect dopaminergic cells more adversely. Therefore, the role of dopamine (DA) in rotenone-induced PC12 cell toxicity was examined. Rotenone (1.0 μ*M*) caused significant toxicity in differentiated PC12 cells, which was accompanied by decreases in ATP levels, changes in catechol levels, and increased DA oxidation. To determine whether endogenous DA makes PC12 cells more susceptible to rotenone, cells were treated with the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine (AMPT) to reduce DA levels prior to rotenone exposure, and then cell viability was measured. No changes in rotenone-induced toxicity were observed with or without AMPT treatment. However, a potentiation of toxicity was observed following coexposure of PC12 cells to rotenone and methamphetamine. To determine whether this effect was due to DA, PC12 cells were depleted of DA prior to methamphetamine and rotenone cotreatment, resulting in a large attenuation in toxicity. These findings suggest that DA plays a role in rotenone-induced toxicity and possibly the vulnerability of DA neurons in PD. *Antioxid. Redox Signal.* 7, 630–638.

INTRODUCTION

THE CAUSE OF SELECTIVE DOPAMINERGIC DEGENERATION in the substantia nigra of Parkinson's disease (PD) patients remains unknown; however, research has linked cell death in PD to oxidative stress (for review, see 10) and mitochondrial dysfunction (6, 43, 44). Direct effects of reactive oxygen species (ROS), including increased lipid peroxidation, protein carbonyls, and DNA damage in PD brain, have been observed (2). Increased iron and significant decreases in the major antioxidant, glutathione (GSH), which also promotes oxidative stress, have also been observed in PD (25).

The relationship between oxidative stress and dopamine (DA) oxidation in degeneration has provided a link between the selective vulnerability of DA neurons and PD. Increased DA metabolism, by both monoamine oxidase (MAO) and DA oxidation into DA quinone (DAQ), will cause increased ROS production, which may lead to oxidation of protein, DNA, and lipids (16, 18, 20, 32). In addition to ROS, the electron-deficient DAQ readily reacts with cellular nucleophiles, including reduced sulfhydryl groups, located on free cysteine residues, GSH, and proteins (14, 15, 56). Modification of free thiols and GSH can lead to the reduction in the amount of antioxidants available to protect the cells from oxidative stress. In addition, free cysteinyl-DA conjugates can be further oxidized to form 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (DHBT-1), a mitochondrial toxin (29). DAQ modification of proteins lead to the formation of covalently bound DA-protein conjugates, often on cysteinyl residues (21). Many vital proteins contain cysteine residues at their active sites, and therefore modification may alter the function of these proteins, leading to inactivation and possibly cell death. Both *in vitro* (16) and *in vivo* (13, 22) studies support the hypothesis that exposure to DA increases protein cysteinyl-catechol levels, and *in vivo* causes selective damage to DA terminals (39). Protein modification by DAQ has also been observed following the

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dopaminergic toxins methamphetamine (METH) (28) and 1-methyl-4-phenylpyridinium (MPP^+) (55), indicative of endogenous DA oxidation. The presence of neuromelanin in the brain and cysteinyl-catechol conjugates in PD brain lysates (53) suggests that DA oxidation occurs *in vivo*. Therefore, the presence of DA in the cytoplasm, especially in a reduced antioxidant environment, will add to the oxidative stress of a cell through ROS and DAQ production and through the subsequent oxidation of important biomolecules, making dopaminergic neurons in the substantia nigra more susceptible to cell death.

Impairment of mitochondrial function is also likely to contribute to oxidative stress and cell death in PD. The link between Complex I inhibition and PD was first identified after the dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was discovered to be a potent Complex I inhibitor. Further studies revealed that impaired mitochondrial function, in the form of a Complex I deficiency, occurs in PD in the substantia nigra (35, 42) and systemically in platelets and muscle (6, 52). The role of Complex I inhibition in PD has been expanded through experimentation with pesticides and toxins that inhibit Complex I, including MPTP, paraquat, and rotenone, all of which cause selective DA degeneration (for reviews, see 11, 12, 17). The selective toxicity of both MPTP and paraquat is due to their similar structure, which makes them substrates for the DA transporter (DAT) (34, 37, 51). Rotenone, however, is not a substrate for DAT. It is lipophilic and can cross membranes of all cells easily. *In vivo* studies have shown that chronic, systemic administration of rotenone produces dopaminergic degeneration and Lewy body-like cytoplasmic inclusions, which closely mimic the pathology of PD (5), although less selective effects have also been observed (23). The systemic rotenone model does represent both the central and peripheral inhibition of Complex I as seen in PD, which leads to nigrostriatal dopaminergic degeneration (5), α-synuclein aggregation (48), and glial activation (49). Rotenone treatment also functions as an effective PD model *in vitro*, resulting in toxicity to dopaminergic cells (19), increasing oxidative stress (47), and decreasing proteasome activity (46).

Partial inhibition of Complex I has been shown to increase mitochondrial production of ROS (38, 57), which may be the precipatory event in toxicity models. However, the basis for rotenone-induced selective toxicity to dopaminergic neurons remains ambiguous. The increased oxidative stress within dopaminergic neurons, due to DA metabolism and oxidation, combined with a Complex I inhibition-induced ROS production may lead to cell death by overloading the oxidative capacity of dopaminergic cells. Therefore, in this study we sought to investigate whether DA was involved in rotenone-induced toxicity in PC12 cells. We found that DA depletion prior to toxin exposure did not protect against rotenone-induced toxicity. However, rotenone toxicity was potentiated by METH-induced increases in cytoplasmic DA in PC12 cells.

MATERIALS AND METHODS

Chemicals

Cell culture media, Dulbecco's modified Eagle medium (DMEM; GIBCO brand), fetal bovine serum (HyClone brand), and horse serum (HyClone brand) were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Rotenone was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.), α-methyl-*p*-tyrosine (AMPT) from Fluka (Ronkonkoma, NY, U.S.A.), and nerve growth factor (NGF) from BD Bioscience (San Diego, CA, U.S.A.) and Accurate Chemical (Westbury, NY, U.S.A.). All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

PC12 cell culture

PC12 cells, a rat adrenal pheochromocytoma-derived cell line, were differentiated in DMEM supplemented with 1% fetal bovine serum, 1% horse serum, and 100 ng/ml NGF for 3 days. Cells were then treated with rotenone [dissolved in dimethyl sulfoxide (DMSO)] and/or methamphetamine, in differentiation media for 2–48 h. Control cultures underwent a medium change at the same time as rotenone-treated cultures. Cell viability was determined by cell counting using the trypan blue exclusion method. Vehicle (DMSO) had no effect on cell viability (data not shown).

Depletion of cellular DA

DA levels were depleted using the tyrosine hydroxylase (TH) inhibitor AMPT. AMPT was added to the differentiating media in concentrations of 100, 300, or 1,000 μM. For subsequent experiments in which DA levels were depleted, 1,000 μM AMPT was added 3 days prior to, and then sustained during, rotenone treatment.

Biochemical analysis

For DA and 3,4-dihydroxyphenylacetic acid (DOPAC) measurements, PC12 cells were collected following treatment, and the protein was acid-precipitated in 0.1 *M* perchloric acid and centrifuged at 14,000 *g* for 25 min. An aliquot of the supernatant was extracted with alumina, and injected into an HPLC system containing an ESA (Chelmsford, MA, U.S.A.) Coulochem II coulometric detector (+280 V). Protein cysteinyl catechols [protein cys-DA, cys-DOPAC, and cysteinyl-3,4-dihydroxyphenylalanine (cys-DOPA)] were measured following hydrolysis of protein in 6 *M* HCl containing 1 mg/ml bovine serum albumin, as described previously (21). Hydrolyzed protein samples were extracted with alumina prior to analysis on HPLC with a Waters 460 amperometric detector set at an oxidizing potential of 0.6 V. Peaks for catechols and cysteinyl-catechols were identified and quantified by comparison with standards.

ATP measurement

Following exposure to DA, protein from PC12 cells was precipitated in 2% trichloroacetic acid and centrifuged at 14,000 *g* for 25 min. A luciferase-based assay was used to measure ATP levels in an aliquot of the resulting supernatant (40). A Monolight 3010 luminometer (Pharmingen, San Diego, CA, U.S.A.) was used to measure the light output resulting after an aliquot of diluted cell sample, 30 m*M* HEPES, pH 7.75, and Enlighten luciferase/luciferin reagent (Promega, Madison, WI, U.S.A.) were mixed in a cuvette. Protein amounts were determined by the Bradford assay (7).

Statistical analysis

Differences among group means were determined by ANOVA followed by *post-hoc* Student's *t* test with significance determined at $p < 0.05$.

RESULTS

Rotenone-induced PC12 cell toxicity

Previous studies have shown the mitochondrial Complex I inhibitor rotenone to be toxic to DA-containing cells, such as undifferentiated PC12 cells (19), SH-SY5Y cells (46), and primary mesencephalic cultures (30). To evaluate the susceptibility of differentiated PC12 cells to rotenone toxicity, cell viability was determined using trypan blue exclusion, following 48 h of rotenone exposure at concentrations ranging from 0.5 μM to 20 μM (Fig. 1). Rotenone treatment for 48 h significantly decreased the number of viable cells, from −37% to −70% as compared with time-matched control cells, at all concentrations measured (Fig. 1). Future experiments used either the 0.5 μM or 1 μM rotenone concentration, because these were the lowest concentrations that caused significant amounts of cell death.

Rotenone reduced ATP levels in PC12 cells

Rotenone exposure inhibits mitochondrial Complex I and in part the electron transport chain, potentially reducing ATP synthesis. Previous studies have shown that rotenone treatment in SK-N-MC human neuroblastoma cells led to a dose-dependent loss in ATP (47). Therefore, to determine whether the rotenone concentrations that caused decreases in viability also led to reductions in ATP level, we measured ATP levels in differentiated PC12 cells treated with 1 μM rotenone, for 12–48 h (Fig. 2). We observed significant decreases in ATP levels following 12, 24, and 48 h of rotenone exposure, ranging from −25% to −65% as compared with time-matched control levels (Fig. 2). The greatest depletion occurred after 48 h of rotenone exposure.

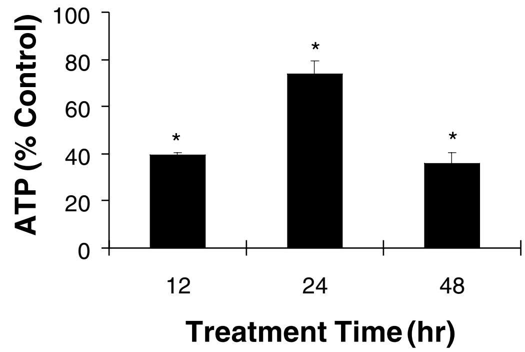

FIG. 2. ATP levels in PC12 cells following rotenone exposure. Differentiated PC12 cells were treated with 1 μM rotenone for 12, 24, or 48 h. ATP levels were measured from PC12 cell lysate in a luminometer. Control ATP levels were 13.0 ± 1.5 nmol/mg of protein. Data are expressed as mean % control ± SEM ($n = 4$). *Statistically significant from control, $p < 0.05$.

Effects of rotenone on catechol levels

Rotenone has previously been shown to cause catecholamine release in PC12 cells (54); therefore, we wanted to determine whether cellular catecholamine levels were affected by rotenone. DOPA, DA, and DOPAC amounts were measured in differentiated PC12 cells treated with 1 μM rotenone for 2–48 h and compared with time-matched control levels (Fig. 3). DOPA levels were significantly increased from control (+130%) following 48 h of 1 μM rotenone treatment. In contrast, DA levels were significantly lower than control following 6–24 h of 1 μM rotenone treatment, ranging from −18% to −25% of time-matched control levels. However, the greatest decrease was observed in DOPAC levels,

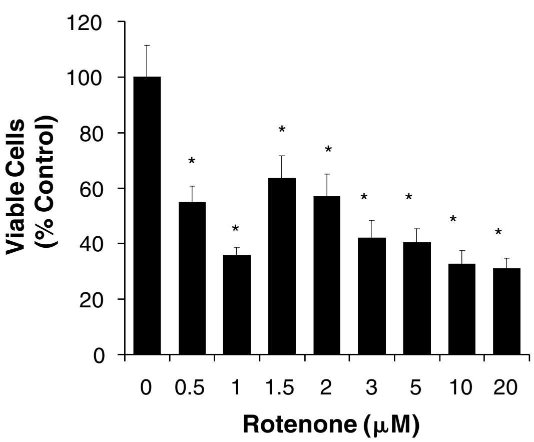

FIG. 1. Concentration curve for rotenone toxicity. Differentiated PC12 cells were treated for 48 h with increasing concentrations of rotenone dissolved in DMSO. The number of viable cells was counted using trypan blue exclusion. Data are expressed as mean % control viable cells ± SEM (n = 3–4). *Significantly different from control, $p < 0.05$.

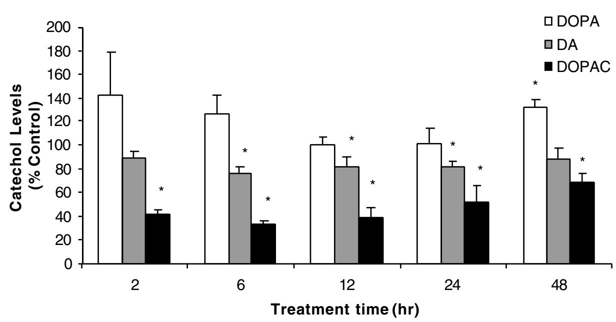

FIG. 3. Catechol levels following rotenone exposure. Differentiated PC12 cells were treated with 1 μM rotenone for 2–48 h. DA, DOPAC, and DOPA levels were determined using HPLC with electrochemical detection. Average control DA levels were 2.0 ± 0.2 nmol/mg of protein. Average control DOPAC levels were 48 ± 4.9 pmol/mg of protein. Average control DOPA levels were 511 ± 51 pmol/mg of protein. Data are expressed as mean % control ± SEM (n = 4–5). *Statistically significant from control, $p < 0.05$.

which were significantly lower (−33% to −68%) than control at all time points observed, following 1 *μM* rotenone treatment. The sustained low levels of DOPAC suggested that rotenone exposure might alter MAO activity, the enzyme that metabolizes DA to DOPAC. To evaluate this possibility, we treated isolated rat brain mitochondria with 1 *μM* rotenone, exposed the mitochondria to 50 *μM* DA, and measured DA metabolites using HPLC. The rates of DA metabolism were similar in rotenone-treated and untreated mitochondria, suggesting that this was not a direct effect of rotenone on MAO activity (data not shown).

Effect of rotenone on DA oxidation

Rotenone inhibits mitochondrial Complex I, leading to ROS production (38, 57). Increased ROS is likely to lead to increased DA oxidation and DAQ formation in DA-containing neurons, which may additionally contribute to the rotenone-induced toxicity. As a measure of DA oxidation and catechol oxidation in general, we evaluated the formation of protein cysteinyl-catechols in PC12 cells treated with 1 *μM* rotenone (Fig. 4). Protein from rotenone-treated cells was acid-precipitated and hydrolyzed to break up the protein into its amino acid components. Protein cysteinyl-DA and cysteinyl-DOPAC levels were then measured using HPLC. Protein cys-DA levels were increased above control (+150%) following 48 h of rotenone treatment (Fig. 4). Protein cys-DOPAC levels were also increased significantly from control (+120–140% of control) after a 12–48-h rotenone treatment (Fig. 4), suggesting that rotenone treatment leads to increased DA and DOPAC oxidation, resulting in protein modification in PC12 cells.

DA depletion does not protect PC12 cells from rotenone-induced toxicity

Because DA may make cells more susceptible to cell death due to the formation of reactive DA metabolites, we examined whether the presence of DA makes PC12 cells more susceptible to rotenone-induced toxicity. To evaluate this initially, DA was depleted in PC12 cells using the TH inhibitor, AMPT. As TH is the rate-limiting step in DA synthesis, blocking TH will stop DOPA, the DA precursor, from being produced, and thus depletes cells of DA and its metabolite, DOPAC. PC12 cells were treated with increasing concentrations of the TH inhibitor, AMPT (0–1,000 *μM*) during the 3-day differentiation period (Fig. 5). Results showed that DA, DOPAC, and DOPA levels were all significantly decreased in a concentration-dependent manner following AMPT treatment (Fig. 5). At the highest concentration (1,000 *μM* AMPT), DA levels were only 7.5% of control and DOPAC and DOPA levels were non-detectable. AMPT (1,000 *μM*) treatment alone had no effect on PC12 cell viability (Fig. 6), and therefore was chosen for all subsequent DA depletion experiments.

The effect of DA depletion on rotenone toxicity was determined by pretreatment with 1,000 *μM* AMPT for 72 h, followed by 1 *μM* rotenone or vehicle plus AMPT for an additional 48 h. Rotenone treatment alone led to a 60% decrease in viable cells. However, AMPT plus rotenone showed a similar decrease in cell viability (−60%), which did not differ from the rotenone-alone treated cells (Fig. 6).

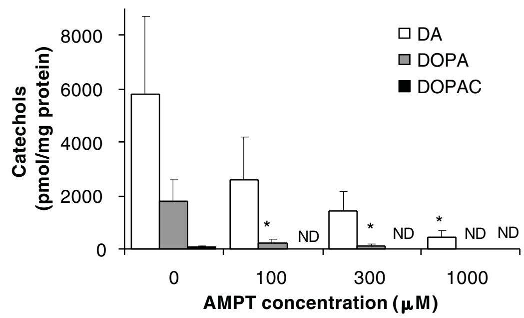

FIG. 5. Effect of AMPT on catechol levels. PC12 cells were treated with 0–1,000 *μM* AMPT, a TH inhibitor, for 72 h during differentiation. DA, DOPAC, and DOPA levels were measured using HPLC with electrochemical detection. Data are expressed as means ± SEM ($n = 4$). *Statistically significant from control, $p < 0.05$.

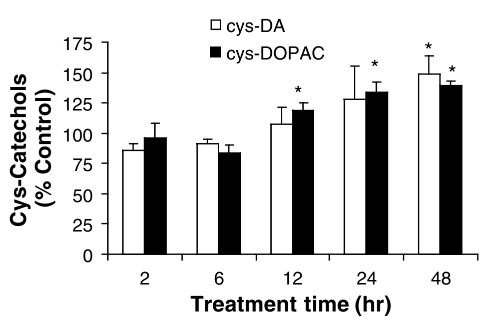

FIG. 4. Protein cysteinyl-catechol levels following rotenone exposure. Differentiated PC12 cells were treated with 1 *μM* rotenone for 2–48 h. Protein cysteinyl-DA and protein cysteinyl-DOPAC levels were determined using HPLC with electrochemical detection. Data are expressed as mean % control ± SEM ($n = 4$–6). *Statistically significant from control, $p < 0.05$.

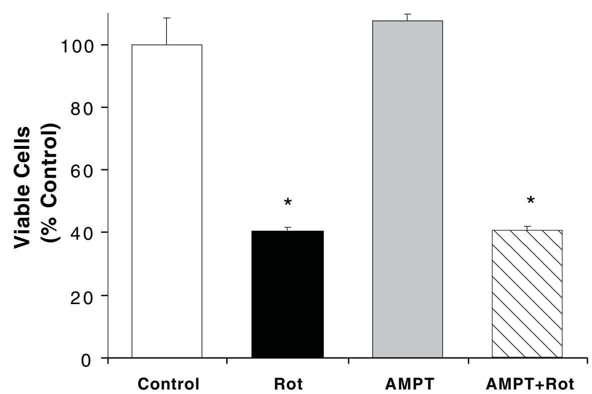

FIG. 6. PC12 cell viability following DA depletion and rotenone exposure. PC12 cells were treated with 1 m*M* AMPT or control medium for 72 h, followed by 1 *μM* rotenone or vehicle for 48 h. Cell viability was then determined using trypan blue exclusion. Data are expressed as mean % control ± SEM ($n = 4$). *Statistically significant from control, $p < 0.05$.

METH potentiated rotenone-induced toxicity in PC12 cells

PC12 cells contain both synaptic-like vesicles and large dense-core vesicles (36), which comprise a large storage capacity for DA, and thus, much of the DA would be adequately sequestered away from any rotenone-produced ROS. Because DA depletion did not attenuate rotenone-induced toxicity, we sought to determine whether increasing cytoplasmic DA by treatment with METH would potentiate rotenone-induced toxicity. Previous studies in primary cultures have shown that rotenone potentiated toxicity induced by amphetamine, which releases DA from vesicles into the cytoplasm (31). First, to confirm the mobilization of intracellular DA stores in PC12 cells following METH treatment, we exposed differentiated PC12 cells to control medium, 0.5 μM rotenone, 0.5 mM METH, or 0.5 μM rotenone plus 0.5 mM METH in medium for 24 h and then measured cellular catechol levels (Fig. 7A). METH treatment alone did not affect DOPA levels. However, there was a significant increase in DOPA levels to 217% and 310% of control in the rotenone- and rotenone plus METH-treated cells, respectively. DOPAC levels were decreased to −55%, −24%, and −72% as compared with control following METH, rotenone, and rotenone plus METH treatment, respectively. DA levels were not affected by rotenone treatment alone, but were significantly decreased −83% from control following 24 h of METH, and −73% after a rotenone plus METH treatment. These data indicate that exposure to METH, but not rotenone, is responsible for mobilizing intracellular DA stores in PC12 cells, leading to DA depletion.

After confirming that METH was mobilizing DA stores, we examined the effect of increased cytosolic DA on rotenone-induced toxicity. We cotreated PC12 cells with 0.5 mM METH and 0.5 μM rotenone, for 48 h, and measured cell viability (Fig. 7B). In these studies, we utilized a lower concentration of rotenone than in previous experiments, which led to a 22% loss in viable cells. METH treatment alone also led to a small, but significant decrease in cell viability (−12% of control). However, cotreatment of rotenone and METH led to a 49% loss in cell viability, which was significantly different from control, rotenone alone, and METH alone groups. In addition, rotenone plus METH appeared to potentiate toxicity beyond the sum of the toxicities seen in either treatment group alone.

To determine whether the effect was due to increased cytosolic DA or due to a direct effect of METH, we first depleted DA with AMPT and then treated PC12 cells with METH and rotenone for 48 h. In this experiment, 0.5 mM METH exposure led to only a 7% loss in viability, which was not significantly different from control (Fig. 8). Rotenone exposure (0.5 μM) led to a 29% loss in cell viability as compared with control, which was again (as in Fig. 7) not significantly different from rotenone treatment following DA depletion with AMPT (−34% as compared with control) (Fig. 8). METH and rotenone cotreatment led to a 46% loss in cell viability as compared with control, which was again signifi-

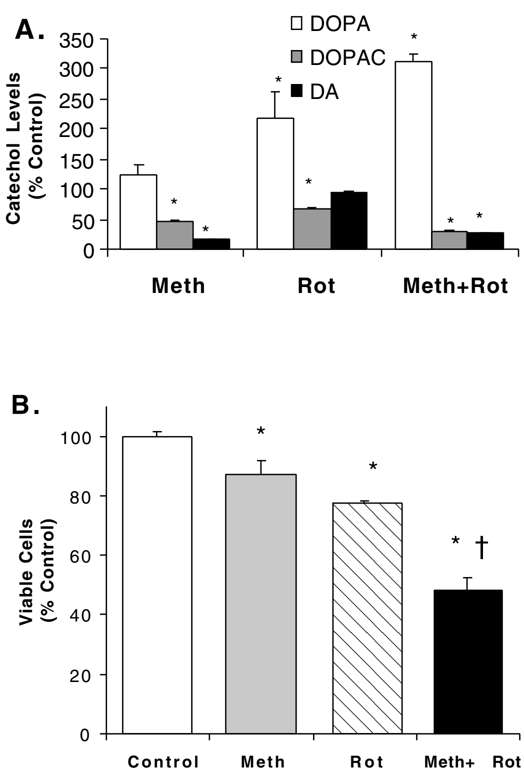

FIG. 7. Effect of METH and rotenone cotreatment on PC12 cell catechol levels and viability. PC12 cells were treated with control medium, 0.5 mM METH, 0.5 μM rotenone, or cotreated with 0.5 μM rotenone and 0.5 mM METH for 48 h. (**A**) DA, DOPAC, and DOPA levels were determined using HPLC with electrochemical detection. Data are expressed as mean % control ± SEM (n = 4). *Statistically significant from control, $p < 0.05$. (**B**) Cell viability was determined using trypan blue exclusion. Data are expressed as mean % control ± SEM (n = 4). *Statistically significant from control, $p < 0.05$. †Statistically significant from METH- and rotenone-alone treated groups, $p < 0.05$.

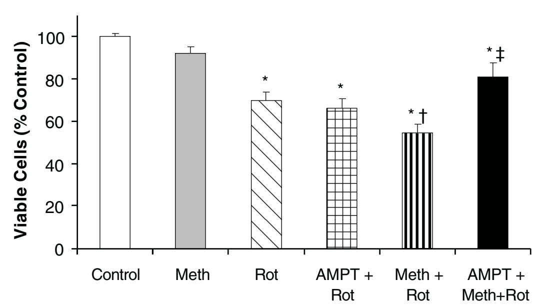

FIG. 8. Effect of DA depletion on METH and rotenone cotreatment on PC12 cell viability. PC12 cells were treated with control medium, 0.5 mM METH, 0.5 μM rotenone, pretreated with 1 mM AMPT for 3 days followed by 0.5 μM rotenone treatment, cotreated with 0.5 μM rotenone and 0.5 mM METH, or pretreated with 1 mM AMPT for 3 days followed by 0.5 μM rotenone and 0.5 mM METH cotreatment for 48 h. Cell viability was then determined using trypan blue exclusion. Data are expressed as mean % control ± SEM (n = 4). *Statistically significant from control, $p < 0.05$. †Statistically significant from METH- and rotenone-alone treated groups, $p < 0.05$. ‡Statistically significant from METH- and rotenone-cotreated group, $p < 0.05$.

cantly different from control, rotenone alone, and METH alone treated groups (Fig. 8). However, 1,000 μM AMPT pretreatment followed by rotenone and METH cotreatment led to attenuation of toxicity to only a 19% loss in viable cells, which represents the rescue of 60% of the cells lost following rotenone plus METH without pretreatment (Fig. 8). This observation suggests that a large portion of the enhanced toxicity observed in rotenone/METH-induced toxicity could be due to the presence of DA.

DISCUSSION

In the present study, we wanted to determine the role of DA in rotenone-induced toxicity, to understand better the possible contribution of DA to cell death in PD. Oxidative stress and mitochondrial dysfunction, combined with DA oxidation, may make dopaminergic cells a more vulnerable target for toxic stimuli in PD. The rotenone model, with Complex I inhibition and selective dopaminergic cell death, possesses many aspects of PD pathology, including evidence of increased oxidative stress (47) and α-synuclein-positive protein aggregates (48). Therefore, we used rotenone, in conjunction with AMPT and METH, to examine the contribution of DA to rotenone-induced toxicity in PC12 cells. We found that DA depletion prior to toxin exposure did not protect against rotenone-induced toxicity. However, rotenone toxicity was potentiated in PC12 cells by the intracellular release of DA from the vesicles, induced by METH exposure.

Partial inhibition of Complex I has been shown to increase mitochondrial production of ROS (38, 57), which may be the precipitating event in toxicity models. However, the basis for rotenone-induced selective toxicity to dopaminergic neurons remains ambiguous. The increased oxidative stress within dopaminergic neurons, due to DA metabolism and oxidation, combined with enhanced ROS production by Complex I inhibition may lead to cell death by overloading the antioxidant capacity of these cells. In addition, DA oxidation may cause mitochondrial dysfunction, because isolated mitochondria exposed to DAQ have increased state 4 (uncoupled) respiration and opening of the permeability transition pore (4). DA oxidation, mitochondrial dysfunction, and mitochondrial ROS production are all processes that can lead to an increasing cascade of oxidative damage to cellular macromolecules, which may lead to total mitochondrial failure and cell death.

We found that the depletion of DA by AMPT did not protect PC12 cells from rotenone-induced toxicity. However, coexposure of PC12 cells to rotenone and METH, which leads to the release of DA stores into the cytoplasm, led to increased toxicity. Additionally, the potentiation of rotenone toxicity by METH was blocked when PC12 cells were depleted of DA prior to rotenone and METH cotreatment. Although METH may have toxic actions on its own (8), these data suggest that cytoplasmic DA, and perhaps increased oxidative stress due to DA oxidation and metabolism, may exacerbate rotenone-induced toxicity in differentiated PC12 cells.

Previous studies in primary mesencephalic cultures (41), SH-SY5Y cells (46), and undifferentiated PC12 cells (19) have shown nanomolar concentrations of rotenone to be toxic. However, in this study, we found a 37–70% decrease in cell viability in NGF-differentiated PC12 cells following a 48-h exposure to 0.5–20 μM rotenone (Fig. 1), and very little toxicity prior to 48 h (data not shown). Differentiated PC12 cells seem to be less susceptible to rotenone-induced toxicity than other cellular models, which may be due to the presence of the growth factor NGF throughout exposure.

ATP levels were depleted following 1 μM rotenone exposure (Fig. 2). However, the levels of ATP after 24 h of rotenone treatment (75% of control) were higher than the ATP levels following 12 h (40% of control) or 48 h (35% of control) of rotenone exposure. The jump in ATP levels may be the result of glycolysis stimulated by rotenone-induced Complex I inhibition. PC12 cells have previously been shown to resort to increased glycolysis when challenged with the mitochondrial Complex I inhibitor, MPP^+ (26). This has also been observed in primary neuronal cultures in response to oxidative stress (3). Our observations suggest that the complete loss of ATP was not responsible for cell death, as also determined by others (50).

Rotenone-induced catecholamine release in PC12 cells has been previously observed (54), and in our study, we observed a slight, but significant, depletion of DA following a 6–24-h treatment with 1 μM rotenone (Fig. 3). However, at 24 h, a lower concentration of rotenone (0.5 μM) did not affect DA levels (Fig. 7A). Therefore, if DA is being released from PC12 cells following rotenone treatment, it is very small compared with the total DA stored in the cells. We also observed a substantial decrease in DOPAC levels in PC12 cells treated with rotenone (Figs. 3 and 7A), but found no direct effect of rotenone on MAO activity in isolated mitochondria. Decreased DOPAC levels have been previously observed in PC12 cells following rotenone treatment (27). Decreased DOPAC following rotenone in that study was accompanied by increased levels of 3,4-dihydroxyphenylacetaldehyde (DOPAL), suggesting that rotenone leads to the inactivation of aldehyde dehydrogenase, the enzyme that converts DOPAL into DOPAC (27). DOPAL exposure has been shown previously to be toxic to dopaminergic cells (33), and thus may add to the rotenone-induced toxicity. However, we did not observe the presence of DOPAL in PC12 cells following rotenone treatment. We also observed increased DOPA levels following rotenone treatment (Figs. 3 and 7A). DOPA-induced toxicity has previously been shown in PC12 cells (1). Like DA, DOPA can oxidize, forming ROS and DOPA quinones, which could add to the oxidative damage in the cell (16).

Rotenone has been shown to increase oxidative stress. In previous cell culture studies, rotenone reduced GSH levels (45, 47), while increasing levels of oxidized glutathione (GSSG) (45). In addition, acute and chronic rotenone exposure in SK-N-MC cells leads to increased carbonyl formation (47, 50). In this study, we observed evidence of increased DA oxidation following rotenone treatment in PC12 cells, as levels of protein cysteinyl-DA and cysteinyl-DOPAC increased after 12–48 h of rotenone exposure (Fig. 4), suggestive of an oxidative environment in the cells.

Previous studies have shown that depletion of DA is protective in MPP^+-induced toxicity (30), and recent studies in primary mesencephalic cultures have suggested that DA may be involved in rotenone-induced toxicity (41). In addition, rotenone potentiated amphetamine-induced toxicity in primary mesencephalic cultures, which was also thought to be due to DA (31). We found that DA depletion did not affect rotenone-induced toxicity in PC12 cells (Fig. 6). However, as DA may not have been accessible for oxidation due to PC12 cell's high storage capacity, we utilized a way to mobilize endogenous DA stores in the presence of rotenone, to determine whether DA could play a role in rotenone-induced toxicity. Previous studies have shown that METH is transported into cells by DAT (15), where it displaces vesicular DA into the cytoplasm (9), leading to DA depletion (15). In PC12 cells, METH treatment and rotenone/METH cotreatment led to DA depletion (Fig. 7A), suggesting that DA was being released from the vesicles into the cytoplasm, where it could be easily oxidized, metabolized, and/or released from the cell via reversal of DAT. Results showed that DA potentiates rotenone-induced toxicity following the mobilization of DA by METH (Fig. 7B), an effect that was eliminated with prior DA depletion (Fig. 8). METH has also been shown to enhance 3-nitropropionic acid and glutamate toxicity (for review, see 24), an effect thought to be dependent on DA. Although oxidative stress is likely to be involved, the mechanism may be different from the intracellular effects on DA neurons.

Rotenone and other Complex I inhibitors are currently being used as PD models both *in vivo* and *in vitro* (17). However, the question of why Complex I inhibitors seem to target dopaminergic neurons has remained unanswered. The present study demonstrates that unsequestered, intracellular DA could play a significant role in the selective targeting of DA neurons in rotenone-induced toxicity. The ability of a dopaminergic cell to deal with increased oxidative stress, created by Complex I inhibition, may be hampered by the presence of DA, which may further increase oxidative stress. The Complex I deficiency observed in PD is likely to cause increased ROS production, which in turn will promote DA oxidation, leading to a cycle of increasing oxidative stress and further DA oxidation. This will result in oxidative protein modifications, inactivation of critical protein functions, and/or altered protein degradation, all of which are likely to contribute to the pathological mechanisms involved in PD.

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ABBREVIATIONS

AMPT, *α*-methyl-*p*-tyrosine; DA, dopamine; DAQ, dopamine quinone; DAT, dopamine transporter; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; DOPA, 3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPAL, 3,4-dihydroxyphenylacetaldehyde; GSH, glutathione; MAO, monoamine oxidase; METH, methamphetamine; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NGF, nerve growth factor; PD, Parkinson's disease; ROS, reactive oxygen species; TH, tyrosine hydroxylase.

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