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Monoamine Oxidase Inhibitors Attenuate Cytotoxicity of 1-Methyl-4-phenylpyridinium by Suppressing Mitochondrial Permeability Transition

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Mitochondrial permeability transition has been shown to be involved in neuronal cell death. Mitochondrial monoamine oxidase (MAO)-B is considered to play a part in the progress of nigrostriatal cell death. The present study examined the effect of MAO inhibitors against the toxicity of 1-methyl-4-phenylpyridinium (MPP^+) in relation to the mitochondrial permeability transition. Chlorgyline (a selective inhibitor of MAO-A), deprenyl (a selective inhibitor of MAO-B) and tranylcypromine (non-selective inhibitor of MAO) all prevented cell viability loss, cytochrome *c* release, caspase-3 activation, formation of reactive oxygen species and depletion of GSH in differentiated PC12 cells treated with 500μM MPP^+. The MAO inhibitors at 10μM revealed a maximal inhibitory effect and beyond this concentration the inhibitory effect declined. On the basis of concentration, the inhibitory potency was tranylcypromine, deprenyl and chlorgyline order. The results suggest that chlorgyline, deprenyl and tranylcypromine attenuate the toxicity of MPP^+ against PC12 cells by suppressing the mitochondrial permeability transition that seems to be mediated by oxidative stress.

Key Words: Monoamine oxidase inhibitors, MPP^+, Mitochondrial permeability transition, Cell death, Preventive effect

INTRODUCTION

It has been shown that the membrane permeability transition of mitochondria is involved in a variety of toxic and oxidative forms of cell injury as well as apoptosis (Crompton, 1999). Much evidence indicates that formation of the mitochondrial permeability transition plays a critical role in the cytotoxicity of parkinsonian neurotoxin 1-methyl-4-phenylpyridinium (MPP^+). Neuronal cell death due to MPP^+ is mediated by opening of the mitochondrial permeability transition pore, release of cytochrome *c*, and activation of caspases (Cassarino et al, 1999; Lotharius et al, 1999; Lee et al, 2000; Lee et al, 2005). Implication of oxidative stress in the MPP^+ toxicity has been demonstrated by that infusion of MPP^+ into the brains of mice and rats increases the formations of lipid peroxides and hydroxyl radicals in the striatum (Rojas & Rios, 1993; Obata, 2002). In contrast, MPP^+ does not induce lipid peroxidation in PC12 cells and antioxidants do not prevent the MPP^+-induced decrease in [^{3}H] dopamine uptake in cells (Fonck & Baudry, 2001). It is therefore uncertain still whether the cytotoxicity of MPP^+ is mediated by increased formation of reactive oxygen species.

Mitochondrial monoamine oxidase (MAO)-B is considered to play a part in the progress of nigrostriatal cell death. R-(-)-deprenyl, an inhibitor of MAO-B, suggested to provide a beneficial effect in the treatment of Parkinson's disease (Birkmayer et al, 1985). However, deprenyl reveals an inconsistent effect against the neurotoxicity due to the toxins glutamate, dopamine and MPP^+ (Maher & Davis, 1996; Vaglini et al, 1996; Lai & Yu, 1997; Andoh et al, 2005). Furthermore, unlike deprenyl, other MAO-B inhibitors including pargyline do not reveal a protective effect against the toxicity of MPP^+ in Vivo and Vitro (Le et al, 1997; Wu et al, 2000). These results suggest that deprenyl may provide a protective effect on neuronal cells against the MPP^+ toxicity independent of the inhibition of MAO-B. It has been shown that the alteration of MAO-A function may affect neuronal cell death in neurodegenerative disorders (Yi et al, 2006). Nevertheless, compared with the reports related to the MAO-B function, the effect of MAO-A inhibition against the neurotoxicity remains uncertain. Chlorgyline, a selective MAO-A inhibitor, is demonstrated to reduce formation of the mitochondrial permeability transition by tyramine oxidation (Marcocci et al, 2002). N-Methylated β-carbolines, which exert an non-selective MAO inhibition, reduce the toxic effects of 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine

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ABBREVIATIONS: carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; $DCFH_2$-DA, 2',7'-dichlorofluorescin diacetate; deprenyl, R-(-)-deprenyl; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); MAO, monoamine oxidase; Mn-TBAP, Mn (III) tetrakis (4-benzoic acid) porphyrin chloride; MPP^+, 1-methyl-4-phenylpyridinium, MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

(Lee et al, 2000).

Although the MAO-B inhibition is suggested to provide a neuroprotective effect, deprenyl reveals an inconsistent effect against the neurotoxicity. Moreover, the protective effect may be accomplished without intervention of MAO-B inhibition. Furthermore, the effect of MAO-A on the neurotoxin-induced cell death remains uncertain. The current study examined the effect of MAO inhibition (chlorgyline, deprenyl and tranylcypromine) against the toxicity of MPP^+ in differentiated PC12 cells in relation to the mitochondria-mediated cell death process and oxidative stress.

METHODS

Materials

Quantikine® M rat/mouse cytochrome *c* assay kit was purchased from R&D systems (Minneapolis, MN, USA), ApoAlert™ CPP32/caspase-3 assay kit from CLONTECH Laboratories Inc. (Palo Alto, CA, USA), R-(-)-deprenyl from RBI (Natick, MA, USA), and Mn (III) tetrakis (4-benzoic acid) porphyrin chloride (Mn-TBAP) from OXIS International Inc. (Portland, OR, USA). 1-Methyl-4-phenylpyridinium (MPP^+), chlorgyline, deprenyl, tranylcypromine, N-acetylcysteine, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'- dichlorofluorescin diacetate (DCFH2-DA), glutathione (GSH, reduced form), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), NADPH, glutathione reductase, phenylmethylsulfonylfluoride (PMSF) and RPMI were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture

Rat PC12 cells (adrenal gland; pheochromocytoma) were obtained from Korean cell line bank (Seoul, Korea). PC12 cells were cultured in RPMI medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin as described in the manual of the cell line bank. Cells were differentiated by treating with 100 ng/ml 7S nerve growth factor for 9 days (Tatton et al, 2002). Cells were washed with RPMI medium containing 1% fetal bovine serum 24 h before experiments and replated onto the 96- and 24-well plates.

Cell viability assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). PC12 cells (4×10^4 cells/200μl) were treated with MPP^+ for 24 h at 37°C. The medium was incubated with 10μl of 10 mg/ml MTT solution for 2 h. After centrifugation at 412×g for 10 min, the culture medium was removed and 100μl dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunnyvale, CA). Cell viability was expressed as a percentage of the value in control cultures.

Measurement of cytochrome c release

The release of cytochrome *c* from mitochondria into the cytosol was assessed by using a solid phase, enzyme-linked immunosorbent assay kit and Western blot analysis. PC12 cells (5×10^5 cells/ml for ELISA assay and 5×10^6 cells for Western blotting) were harvested by centrifugation at 412 ×g for 10 min, washed twice with PBS, suspended in buffer (in mM): sucrose 250, KCl 10, $MgCl_2$ 1.5, EDTA 1, EGTA 1, dithiothreitol 0.5, PMSF 0.1 and HEPES-KOH 20 at pH 7.5 and homogenized further by successive passages through a 26-gauge hypodermic needle. The homogenates were centrifuged at 100,000×g for 30 min and the supernatant was used for analysis of cytochrome *c*. The supernatants and cytochrome *c* conjugate were added into the 96-well microplates coated with monoclonal antibody specific for rat/mouse cytochrome *c*. The procedure was performed according to the manufacturer's instructions. Absorbance of samples was measured at 450 nm in a microplate reader. A standard curve was constructed by adding diluted solutions of cytochrome *c* standard, handled like samples, to the microplates coated with monoclonal antibody. The amount was expressed as nanograms/ml by reference to the standard curve.

For Western blotting, Supernatants were mixed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min. Samples (30 μg/ml protein) were loaded onto each lane of 12% SDS-polyacrylamide gel and transferred onto PVDF membranes (Amersham Biosciences Co., Piscataway, NJ, USA). Membranes were blocked for 2 h in TBS (50 mM Tris-HCl, pH 7.5 and 150 mM NaCl) containing 0.1% Tween 20 and 5% non-fat dried milk. The membranes were labeled with anti-cytochrome *c* (diluted 1 : 1000 in TBS containing 0.1% Tween 20) overnight at 4°C with gentle agitation. After four washes in TBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1 : 2,000) for 2 h at room temperature. Protein bands were identified with the enhanced chemiluminescence detection using SuperSignala® West Pico chemiluminescence substrate.

Measurement of caspase-3 activity

PC12 cells (2×10^6 cells/ml) were treated with MPP^+ for 24 h at 37°C and caspase-3 activity was determined according to the user's manual for the ApoAlert™ CPP32/Caspase-3 assay kit. The supernatant obtained by a centrifugation of lysed cells was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (N-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) and incubated for 1 h at 37°C. Absorbance of the chromophore *p*-nitroanilide produced was measured at 405 nm. The standard curves were obtained from the absorbances of *p*-nitroanilide standard reagent diluted with cell lysis buffer (up to 20 nM). One unit of the enzyme was defined as the activity producing 1 nmol of *p*-nitroanilide.

Measurement of intracellular reactive oxygen species formation

The dye $DCFH_2$-DA, which is oxidized to fluorescent 2',7'-dichlorofluorescin (DCF) by hydroperoxides, was used to measure relative levels of cellular peroxides (Fu et al,

1998). PC12 cells (4×10^4 cells/200μl) were treated with MPP^+ for 24 h at 37°C, washed, suspended in FBS-free RPMI, incubated with 50μM dye for 30 min at 37°C and washed with phosphate buffered saline. The cell suspensions were centrifuged at 412×g for 10 min, and medium was removed. Cells were dissolved with 1% Triton X-100 and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (SPECTRAFLUOR, TECAN, Salzburg, Austria).

Measurement of total glutathione

The total glutathione (reduced form GSH+oxidized form GSSG) was determined using glutathione reductase (van Klaveren et al, 1997). PC12 cells (4×10^4 cells/200μl) were treated with MPP^+ for 24 h at 37°C, centrifuged at 412×g for 10 min in a microplate centrifuge and the medium removed. The pellets were washed twice with phosphate buffered saline, dissolved with 2% 5-sulfosalicylic acid (100 μl) and incubated in 100μl of the reaction mixture containing 22 mM sodium EDTA, 600μM NADPH, 12 mM DTNB and 105 mM NaH_2PO_4, pH 7.5 at 37°C. Glutathione reductase (20μl, 100 units/ml) was added and the mixture incubated for a further 10 min. Absorbance was measured at 412 nm using a microplate reader. The standard curve was obtained from absorbance of the diluted commercial GSH that was incubated in the mixture as in samples.

Statistical analysis

Data are expressed as means±SEM. Statistical analysis was performed by one-way analysis of variance. When significance was detected, post hoc comparisons between the different groups were made using the Duncan's test for multiple comparisons. A probability less than 0.05 was considered to be statistically significant.

RESULTS

Effect of MAO inhibitors on cell viability loss due to MPP^+ exposure

The effect of MAO inhibitors on the MPP^+-induced cell death was assessed using PC12 cells that were differentiated by nerve growth factor. The incidence of cell death after exposure to 500μM MPP^+ for 24 h was about 46%. The addition of chlorgyline (a selective MAO-A inhibitor), deprenyl (a selective MAO-B inhibitor) and tranylcypromine (non-selective MAO inhibitor) up to 25μM significantly attenuated the MPP^+-induced cell death. MAO inhibitors had a maximum inhibition on cell death at 10 μM; beyond this concentration the inhibitory effect declined (Fig. 1). At the 10μM concentration, the inhibitory potency was tranylcypromine (47%), deprenyl (35%) and chlorgyline (23%) order. To assess the cytotoxic effect of MAO inhibitors alone, PC12 cells were treated with the compounds in the absence of MPP^+ for 24 h. As shown in the data, they at the concentration of 25μM caused 5～8% cell viability loss.

Effect of MAO inhibitors on cytochrome c release and activation of caspase-3 due to MPP^+ exposure

Opening of the mitochondrial permeability transition pore

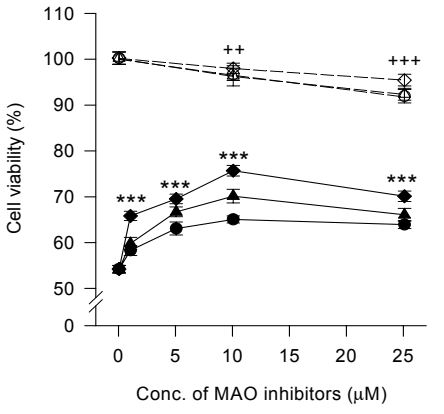

Fig. 1. Inhibition of MPP^+-induced cell death by MAO inhibitors. PC12 cells were pre-treated with MAO inhibitors (1～25μM of chlorgyline, deprenyl and tranylcypromine) for 15 min, exposed to 500μM MPP^+ for 24 h, and cell viability was determined. Data represent means±SEM (n=6), and n indicates the number of experimental case. $^+p<0.05$ compared to control (percentage of control) and $*p<0.05$ compared to MPP^+ alone. Chlorgyline (- ● -), deprenyl (- ▲ -) and tranylcypromine (- ◆ -) in the presence of MPP^+; chlorgyline (- ○ -), deprenyl (- △ -) and tranylcypromine (- ◇ -) without MPP^+.

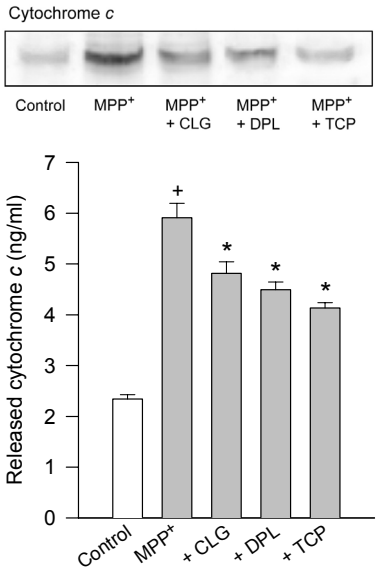

Fig. 2. Preventive effect of MAO inhibitors on release of cytochrome *c* due to MPP^+. PC12 cells were treated with 500μM MPP^+ in the presence of MAO inhibitors [10μM of chlorgyline (CLG), deprenyl (DPL) or tranylcypromine (TCP)] for 24 h. Data are expressed as ng/ml for cytochrome *c* release and represent means±SEM (n=6). $^+p<0.05$ compared to control and $*p<0.05$ compared to MPP^+ alone. The levels of cytochrome *c* in the cytosolic fractions were also analyzed by Western blotting with anti-cytochrome *c* antibody, and data are representative of three different experiments.

causes the release of cytochrome *c* from mitochondria into the cytosol and subsequent activation of caspases as one of the mitochondria-mediated cell death signaling events (Crow et al, 2004; Kim et al, 2006). The cytochrome *c* released from mitochondria induces the activation of caspase-3, an apoptotic factor. The MPP^+-induced change in the mitochondrial membrane permeability was assessed by measuring a release of cytochrome *c* into the cytosol and activation of caspase-3. PC12 cells incubated with 500μM MPP^+ for 24 h revealed a 2.5 fold increase of cytochrome *c* in the cytosol. Chlorgyline, deprenyl and tranylcypromine (all 10μM) significantly prevented the MPP^+-induced release of cytochrome *c* and activation of caspase-3 (Fig. 2, 3). As observed in cell viability, the inhibitory potency of tranylcypromine on the release of cytochrome *c* and activation of caspase-3 was greater than other inhibitors. The inhibitory effect of MAO inhibitors on the MPP^+-induced release of cytochrome *c* was also identified by Western blot analysis (Fig. 2).

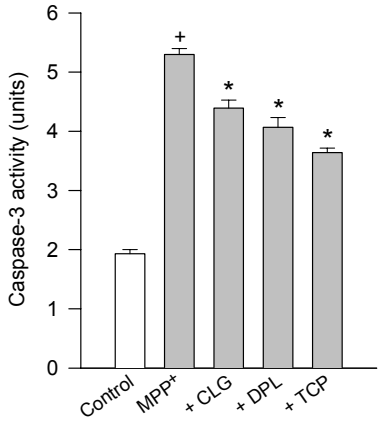

Fig. 3. Depressant effect of MAO inhibitors on activation of caspase-3 due to MPP^+. PC12 cells were treated with 500μM MPP^+ in the presence of MAO inhibitors [10μM of chlorgyline (CLG), deprenyl (DPL) or tranylcypromine (TCP)] for 24 h. Data are expressed as units for caspase-3 activity, and represent means± SEM (n=6). † $p<0.05$ compared to control and *$p<0.05$ compared to MPP^+ alone.

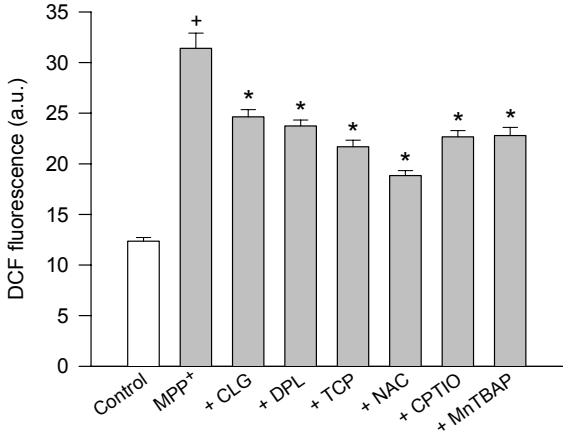

Fig. 4. Inhibition of MPP^+-induced formation of reactive oxygen species by MAO inhibitors. PC12 cells were treated with 500μM MPP^+ in the presence either of MAO inhibitors [10μM of chlorgyline (CLG), deprenyl (DPL) or tranylcypromine (TCP)] or of the scavengers [1 mM N-acetylcysteine (NAC), 25μM carboxy-PTIO (CPTIO) and 30μM Mn-TBAP (MnTBAP) for 24 h. Data are expressed as arbitrary units of fluorescence and represent means± SEM (n=6). † $p<0.05$ compared to control and *$p<0.05$ compared to MPP^+ alone.

Effect of MAO inhibitors on MPP^+-induced formation of reactive oxygen species and depletion of GSH

To determine whether the MPP^+-induced cell death is mediated y oxidative stress, the current study investigated the formation of reactive oxygen species within cells by monitoring a conversion of $DCFH_2$-DA to DCF. Treatment of PC12 cells with 500μM MPP^+ resulted in a significant increase in DCF fluorescence. Chlorgyline, deprenyl and tranylcypromine (all 10μM) significantly inhibited the MPP^+-induced increase in DCF fluorescence (Fig. 4). To confirm further the formation of reactive oxygen species in PC12 cells exposed to MPP^+, the present study examined the inhibitory effect of scavengers. The addition of 1 mM thiol compound N-acetylcysteine, 25μM carboxy- PTIO (a scavenger of nitric oxide) and 30μM Mn-TBAP (a scavenger of peroxynitrite and cell-permeable metalloporphyrin that mimics superoxide dismutase) inhibited the increase in DCF fluorescence due to MPP^+ (Fig. 4).

Drops in cellular GSH levels increase the sensitivity of neurons to the toxic effect of neurotoxins and induce changes in mitochondrial function (Hall, 1999). The work conducted whether the inhibitory effect of MAO inhibitors on the toxicity of MPP^+ was ascribed to the effect on the depletion of GSH. The thiol content in the control PC12 cells was 5.35±0.14 nmol/mg protein. Treatment with 500 μM MPP^+ for 24 h depleted GSH contents by 45%. Chlorgyline, deprenyl and tranylcypromine (all 10μM) significantly prevented the MPP^+-induced depletion of GSH in PC12 cells and showed 27～46% inhibition (Fig. 5). MAO inhibitors alone at 10μM did not cause a significant re-

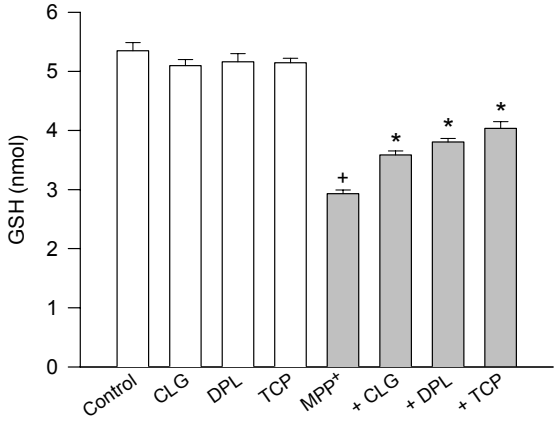

Fig. 5. Preventive effect of MAO inhibitors on MPP^+-induced decrease in the GSH contents. PC12 cells were treated with 500μM MPP^+ in the presence either of MAO inhibitors [10μM of chlorgyline (CLG), deprenyl (DPL) or tranylcypromine (TCP)] for 24 h. Data are expressed as nmol of GSH/mg protein and represent means±SEM (n=6). † $p<0.05$ compared to control and *$p<0.05$ compared to MPP^+ alone.

duction in the GSH contents.

DISCUSSION

The pathologic feature in Parkinson's disease reveals the striking degenerative loss of dopaminergic neurons in the nigrostriatal system. Although rat PC12 cells are not brain dopaminergic neurons, these cells are able to produce dopamine and express dopamine transporter (Kadota et al, 1996). Upon nerve growth factor stimulation, PC12 cells not only display abundant neuritic growth, but also adopt a neurochemical dopaminergic phenotype. On the basis of the character of PC12 cells, the present study assessed the cytotoxicity of MPP^+ against dopaminergic neurons using the PC12 cells that are differentiated by nerve growth factor. The mitochondria- mediated cell death signaling process, results in the activation of caspase-3, is suggested to be involved in the MPP^+-induced apoptosis in neuronal cells (Cassarino et al, 1999; Lotharius et al, 1999; Lee et al, 2002). A significant cytotoxic effect of MPP^+ on cell viability in differentiated PC12 cells was demonstrated by using MTT assay and by the cytochrome *c* release followed by caspase-3 activation, which is involved in apoptotic cell death. In consistent with the previous reports, in this study the MPP^+-induced apoptotic cell death in PC12 cells seems to be mediated by mitochondrial dysfunction, leading to cytochrome *c* release and subsequent activation of caspase-3.

Mitochondrial MAO-B is considered to play a part in the progress of nigrostriatal cell death. It has been proposed that deprenyl exerts a beneficial effect in the treatment of Parkinson's disease through a selective inhibition of MAO-B (Birkmayer et al, 1985). In contrast, there is evidence that the protective effect of deprenyl against the toxic effect of neurotoxins has not been mediated by the inhibition of MAO (Tatton & Chalmers-Redman, 1996; Wu et al, 2000). It has been shown that deprenyl and propargyline increase neuronal survival independently of MAO-B inhibition by interfering with apoptosis signaling pathway (Tatton et al, 2003). The addition of non-selective inhibitors of MAO (harmalol and harmaline) attenuate the neurotoxicity of MPTP on mouse and reveal the inhibitory effect on the loss of the membrane potential due to MPP^+ in isolated brain mitochondria (Lee et al, 2000). As observed in this study, regardless of MAO type selectivity, chlorgyline, deprenyl and tranylcypromine all prevented the MPP^+-induced cell death. Furthermore, non-selective inhibitor tranylcypromine showed an inhibitory effect greater than deprenyl. Therefore, it is more relevant to assess the preventive effect of MAO inhibitors related to the mitochondrial membrane permeability rather than enzyme activity. With respect to the mitochondrial membrane permeability, the current study performed to assess the preventive effect of MAO inhibitors against the cytotoxicity of MPP^+. The results suggest that MAO inhibitors may reduce the MPP^+-induced cell death in differentiated PC12 cells by suppressing mitochondrial damage, leading to cytochrome *c* release and caspase-3.

The MPP^+ treatment causes the respiratory chain inhibition, leading to the formation of reactive oxygen species and nitrogen species (Obata, 2002; Lee et al, 2002). Reactive oxygen species act upon mitochondria, causing a disruption of mitochondrial membrane potential and the release of cytochrome *c* (Chandra et al, 2000). In contrast to these reports, the MPP^+-induced reduction in the [^{3}H] dopamine uptake in PC12 cells is not mediated by formation of reactive oxygen species (Fonck & Baudry, 2001). In the present study, the formation of reactive oxygen species in PC12 cells exposed to MPP^+ and the inhibitory effect of scavengers, including Mn-TBAP and carboxy-PTIO, suggest that MPP^+ induces the formation of reactive oxygen species and nitrogen species in PC12 cells, which is involved in mitochondrial dysfunction. The current study assessed whether the preventive effect of MAO inhibitors on mitochondrial dysfunction come from their interfering effect on oxidative stress. During the apoptotic process, drops in GSH levels and concomitant increase in reactive oxygen species are detected (Tan et al, 1998; Chandra et al, 2000). The oxidation and depletion of GSH modulate opening of the mitochondrial permeability transition pore (Constantini et al, 1996; Hall, 1999). As shown in the results, MAO inhibitors significantly prevented the increased formation of reactive oxygen species and decrease in GSH contents due to MPP^+ exposure. The inhibitory potency of MAO inhibitors on the formation of reactive oxygen species and depletion of GSH correlated with their effect on mitochondrial damage and cell death. These results suggest that chlorgyline, deprenyl and tranylcypromine attenuate mitochondrial damage by decreasing oxidative stress, whose action increases cell survival.

Overall, therefore, the present study concludes that chlorgyline, deprenyl and tranylcypromine may reduce the MPP^+-induced cell viability loss in PC12 cells by suppressing the mitochondrial permeability transition, leading to cytochrome *c* release and caspase-3 activation, which is associated with increased oxidative stress.

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