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Differential Inhibition of MPP^+- or 6-Hydroxydopamine-induced Cell Viability Loss in PC12 Cells by Trifluoperazine and W-7

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The present study assessed the effect of calmodulin antagonists trifluoperazine and W-7 against the cytotoxicity of MPP^+ and 6-hydroxydopamine (6-OHDA) in relation to the mitochondrial dysfunction and cell death in PC12 cells. Trifluoperazine (an inhibitor of the mitochondrial permeability transition and calmodulin antagonist) and W-7 (a specific calmodulin antagonist) significantly attenuated the MPP^+-induced cell viability loss in PC12 cells with a maximum inhibition at 0.5~1 μM; beyond these concentrations the inhibitory effect declined. Both compounds at this concentration range did not cause cell death significantly. In contrast to MPP^+, the trifluoperazine and W-7 did not depress the cytotoxic effect of 6-OHDA. Addition of trifluoperazine and W-7 inhibited the cytosolic accumulation of cytochrome c and caspase-3 activation in PC12 cells treated with MPP^+ and attenuated the formation of reactive oxygen species and the depletion of GSH, whereas both compounds did not reduce the effect of 6-OHDA. The results show that trifluoperazine and W-7 may attenuate the cytotoxicity of MPP^+ by inhibition of the mitochondrial permeability transition and calmodulin. Meanwhile, the cytotoxic effect of 6-OHDA seems to be mediated by the actions, which are different from MPP^+.

Key Words: MPP^+, 6-Hydroxydopamine, Mitochondrial dysfunction, Trifluoperazine, W-7, PC12 cells

INTRODUCTION

The mitochondrial membrane permeability transition is known as a central event in the course of a variety of toxic and oxidative forms of cell injury (Mignotte & Vayssiere, 1998; Crompton, 1999). Neuronal cell death due to 1-methyl-4-phenylpyridium (MPP^+) is mediated by opening of the mitochondrial permeability transition pore, leading to the release of cytochrome c and activation of caspases (Cassarino et al, 1999; Lee et al, 2002). 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 et al, 2002). Meanwhile, the MPP^+-induced decrease in [^{3}H] dopamine uptake in PC12 cells is not prevented by GSH and superoxide scavengers, and MPP^+ does not induce lipid peroxidation (Fonck & Baudry, 2001). It is therefore uncertain whether reactive oxygen species (ROS) is involved in cytotoxicity of MPP^+. The 6-hydroxydopamine (6-OHDA) induces cell death by formation of free radicals and toxic quinones (Galindo et al, 2003; Jordán et al, 2004), but it shows an inconsistent effect on mitochondrial respiratory chain and membrane permeability (Wu et al, 1996; Galindo et al, 2003; Mazzio et al, 2004).

Cyclosporin A (CsA) as an inhibitor of the mitochondrial permeability transition is demonstrated to interfere with the loss of the mitochondrial transmembrane potential and cell death due to glutamate, Ca^{2+} ionophore and ischemic/reperfusion injury (Lemasters et al, 1999; Steinmetz et al, 2004). However, it is uncertain whether toxicities of MPP^+ and 6-OHDA are mediated by CsA-sensitive mitochondrial permeability transition. CsA attenuates the toxicity of rotenone or MPP^+ against PC12 cells (Seaton et al, 1998), but it potentiates MPP^+-induced apoptosis in SH-SY5Y cells (Fall & Bennett, 1998). 6-OHDA-induced injury of dopaminergic neurons in the mouse brain is reduced by CsA (Matsuura et al, 1996), while cell death due to 6-OHDA in chromaffin cells is not mediated by mitochondrial swelling (Galindo et al, 2003). Trifluoperazine has been shown to reduce oxidative stress-induced cell death by suppressing formation of the mitochondrial permeability transition (Shen et al, 2001; Rodrigues et al, 2002). Meanwhile, at high concentrations phenothiazines, including trifluoperazine, show a strong cytotoxicity and antiproliferative activity against neuronal cells and leukemic cells (Gil-Ad et al, 2004; Zhelev et al, 2004).

Although much evidence reveals that MPP^+ and 6-OHDA induce mitochondrial dysfunction, leading to cell death, it is uncertain whether the cytotoxic effect is mediated by the CsA-sensitive permeability transition. MPP^+ and 6-OHDA are suggested to induce neuronal cell death by the differential toxic action (Lotharius et al, 1999; Lee et al, 2002). Calmodulin antagonists such as trifluoperazine and W-7 are demonstrated to reduce oxidative stress-induced apoptotic cell death by inhibiting the mitochondrial dysfunction

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ABBREVIATIONS: MPP^+, 1-methyl-4-phenylpyridium; 6-OHDA, 6-hydroxydopamine; CsA, Cyclosporin A; ROS, reactive oxygen species (ROS).

(Shen et al, 2001; Rodrigues et al, 2002). Nevertheless, the effects on cytotoxicity of MPP^+ and 6-OHDA have not been clarified. The aim of the present study was therefore to assess the effect of trifluoperazine and W-7 against the cytotoxicity of MPP^+ or 6-OHDA in PC12 cells.

METHODS

Reagents

Quantikine® M rat/mouse cytochrome c assay kit was from R&D systems (Minneapolis, MN, USA), ApoAlert™ CPP32/caspase-3 assay kit was purchased from CLONTECH Laboratories Inc. (Palo Alto, CA, USA), and trolox and Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) was from OXIS International Inc. (Portland, OR, USA). 1-Methyl-4-phenylpyridinium iodide (MPP^+), 6-hydroxydopamine (6-OHDA), trifluoperazine, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), 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), 3, 3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), 2',7'-dichlorofluorescin diacetate ($DCFH_2$-DA), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), glutathione reductase, phenylmethylsulfonylfluoride (PMSF) and RPMI 1640 medium were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Protein concentration was determined by the method of Bradford according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

Cell culture

Rat PC12 cells (adrenal gland; pheochromocytoma) were obtained from Korean cell line bank (Seoul, South Korea). PC12 cells were cultured in RPMI medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin as described in the manual of the cell line bank. 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) were treated with neurotoxins for 24 h at 37°C. The medium (200 μl) was incubated with 10 μl of 10 mg/ml MTT solution for 2 h at 37°C. Culture medium was removed, and 100 μl of dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Spectra MAX 340, Sunnyvale, CA, USA). Cell viability was expressed as a percentage of the control culture value.

Measurement of cytochrome c release

The release of cytochrome c from mitochondria into the cytosol was assessed by using a solid phase ELISA kit for the detection of cytochrome c. PC12 cells (5×10^5/ml) were harvested by centrifugation at $412\times g$ 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 needle. The homogenates were centrifuged at $100{,}000\times g$ for 30 min, and the supernatant obtained 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 as described in the assay kit. Absorbance of samples was measured at 450 nm in a microplate reader. A standard curve was constructed by adding the 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.

Measurement of caspase-3 activity

The activation of caspase-3 occurred during the apoptotic process in cells was assessed (Mignotte & Vayssiere, 1998). PC12 cells (2×10^6 cells/ml) were treated with neurotoxins for 24 h at 37°C, and caspase-3 activity was determined as described in user's manual of ApoAlert™ CPP32/ Caspase-3 assay kit. The supernatant obtained by a centrifugation of cells dissolved 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 absorbances in the 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 ROS 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). After exposure to MPP^+, PC12 cells (4×10^4) were incubated with 50 μM dye for 30 min at 37°C and then were washed with PBS. The cell suspensions were centrifuged at $412\times g$ 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) were treated with neurotoxins for 24 h at 37°C, centrifuged at $412\times g$ for 10 min in a microplate centrifuge, and medium was removed. The pellets were washed twice with PBS. Cells were dissolved with 2% 5-sulfosalicylic acid (100 μl) and then incubated in 100 μl of the reaction mixture containing 22 mM sodium EDTA, 600 μM NADPH, 12 mM DTNB and 105 mM NaH_2PO_4, pH 7.5 at 37°C. Glutathione reductase (20 μl, 100 U/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 ob-

tained 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 trifluoperazine and W-7 on the cell viability loss due to MPP^+ or 6-OHDA

To exclude the role of calmodulin in the differentiation of PC12 cells by nerve growth factor, the present study assessed the effect of calmodulin antagonists trifluoperazine and W-7 on cytotoxicity of neurotoxins using undifferentiated PC12 cells. When PC12 cells were treated with 500 μM MPP^+ or 50 μM 6-OHDA for 24 h, the cell viability decreased to 51 and 53%, respectively. Addition of trifluoperazine (an inhibitor of the mitochondrial permeability transition and calmodulin antagonist) and W-7 (a specific calmodulin antagonist) significantly reduced the MPP^+-induced cell death in PC12 cells (Fig. 1A). The maximum inhibitory effect of antagonists against the MPP^+ cytotoxicity was achieved at 0.5 ~ 1 μM; beyond these concentrations the inhibitory effect declined. Meanwhile, both compounds at 10 μM did not show a protective effect because of their cytotoxicity. In contrast to MPP^+, the trifluoperazine and W-7 (0.25 ~ 10 μM) did not inhibit the 6-OHDA-induced cell death at all (Fig. 1B). The present study investigated the cytotoxic effect of calmodulin antagonist alone on PC12 cells. Trifluoperazine and W-7 at 5 ~ 10 μM caused a cell viability loss in PC12 cells (Fig. 2).

To assess whether the cytotoxicity of MPP^+ or 6-OHDA is mediated by oxidative stress, this study examined the inhibitory effect of various antioxidants. Addition of 1 mM thiol compound N-acetylcyteine, 10 μg/ml catalase (a scavenger of hydrogen peroxide), 20 μM trolox (a scavenger of hydroxyl radicals and peroxynitrite), 25 μM carboxy-PTIO (a scavenger of nitric oxide) and 30 μM Mn-TBAP (a scavenger of peroxynitrite and cell-permeable metalloporphyrin that mimics superoxide dismutase) all significantly attenuated the MPP^+-or 6-OHDA-induced viability loss of PC12 cells (Fig. 3).

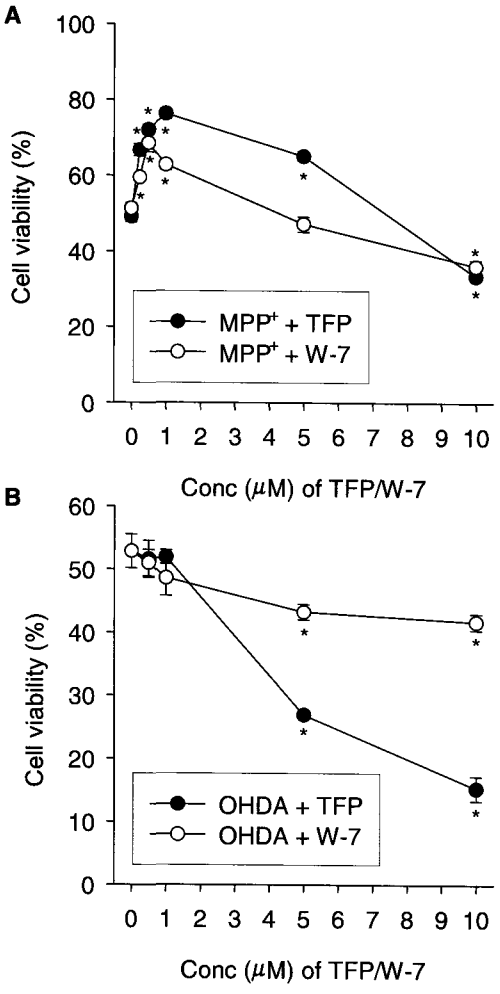

Fig. 1. Effect of trifluoperazine and W-7 on the neurotoxin-induced cell death. PC12 cells were pre-treated with compounds (0.25 ~ 10 μM of trifluoperazine [TFP] and W-7) for 15 min, exposed to 500 μM MPP^+ or 50 μM 6-OHDA for 24 h, and cell viability was determined. Data are expressed as the percentage of cell viability and represent means ± SEM (n=6). *P < 0.05, compared to neurotoxin alone.

Effect of trifluoperazine and W-7 on cytochrome c release and caspase-3 activation

Opening of the mitochondrial membrane permeability transition pore in apoptotic cell death causes the release of cytochrome c from mitochondria into the cytosol, leading to the activation of caspases (Mignotte & Vayssiere, 1998).

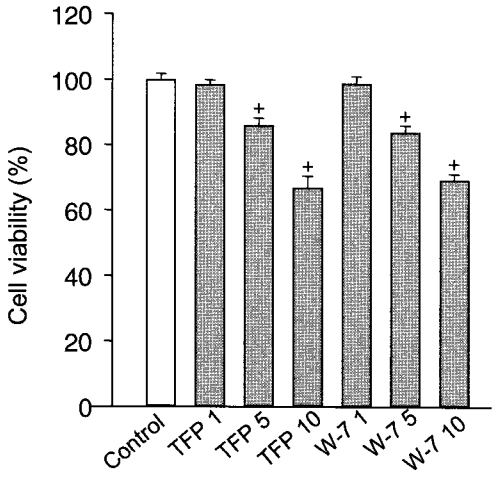

Fig. 2. Cytotoxic effect of trifluoperazine and W-7. PC12 cells were treated with 1 ~ 10 μM of trifluoperazine (TFP) and W-7 for 24 h, and then cell viability was determined. Data are expressed as the percentage of cell viability and represent means ± SEM (n=6). +P < 0.05, compared to control.

Changes in the mitochondrial membrane permeability due to neurotoxins were assessed by measuring a release of cytochrome c into the cytosol and subsequent activation of caspase-3. PC12 cells exposed to MPP^+ exhibited a cytochrome c release and activation of caspase-3. Treatment with 1 μM trifluoperazine or 0.5 μM W-7 significantly reduced the MPP^+-induced cytochrome c release and caspase-3 activation (Fig. 4). Trifluoperazine and W-7 (0.5~1 μM) alone did not induce cytochrome c and caspase-3 activation. Treatment with 6-OHDA also showed the increase in the cytosol of cytochrome c and caspase-3 activation in PC12 cells. However, like the effects on cell death, trifluoperazine and W-7 (0.5~1 μM) did not attenuate the 6-OHDA-induced mitochondrial damage followed by activation of caspase-3.

Effects of trifluoperazine on formation of ROS and depletion of GSH

To determine whether ROS are involved in neurotoxin-induced cell death in PC12 cells, the present study investigated the formation of ROS within cells by monitoring a conversion of $DCFH_2$-DA to DCF. Exposure of PC12 cells to MPP^+ or 6-OHDA for 24 h showed a significant increase in DCF fluorescence, a response that was depressed by the addition of 1 mM N-acetylcysteine. Trifluoperazine (1 μM) and 0.5 μM W-7 significantly attenuated the MPP^+-induced increase in DCF fluorescence, whilst they did not affect the effect of 6-OHDA (Fig. 5).

Reduction of GSH levels increases the sensitivity of neurons to the toxic effect of neurotoxins and is associated with mitochondrial dysfunction (Tan et al, 1998; Chandra

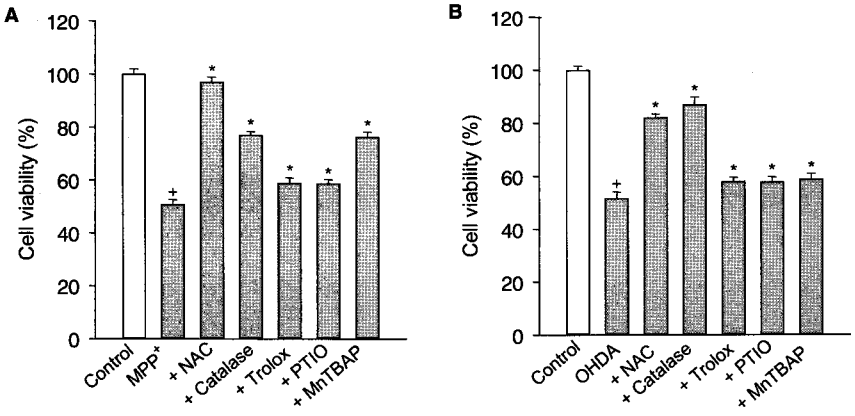

Fig. 3. Effect of antioxidants on the MPP^+- or 6-OHDA-induced cell death. PC12 cells were pre-treated with scavengers (1 mM N-acetylcysteine [NAC], 10 μg/ml catalase, 20 μM trolox, 25 μM carboxy-PTIO [PTIO] and 30 μM Mn-TBAP [MnTBAP]), exposed to 500 μM MPP^+ or 50 μM 6-OHDA, and then cell viability was determined. Data are expressed as the percentage of cell viability and represent means±SEM (n=6). $^+P<0.05$, compared to control. $^*P<0.05$, compared to neurotoxin alone.

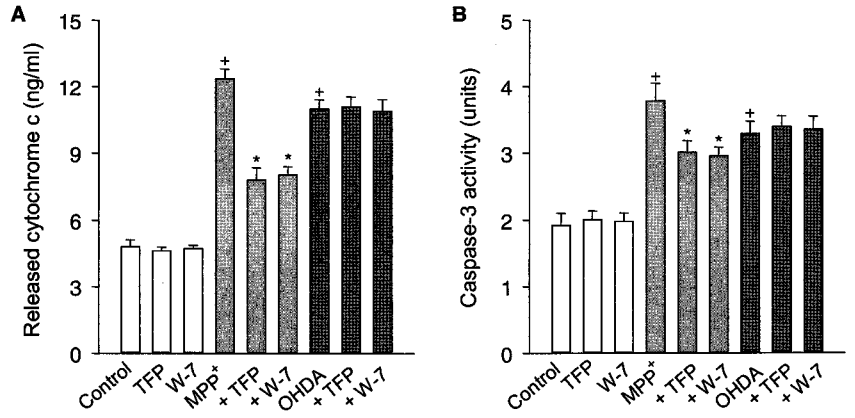

Fig. 4. Effect of trifluoperazine on cytochrome c release and activation of caspase-3. PC12 cells were treated either with neurotoxins in the presence of compounds (1 μM trifluoperazine [TFP] and 0.5 μM W-7) or with calmodulin antagonist alone for 24 h. Data are expressed as nanograms/ml for cytochrome c release (A) and units for caspase-3 activity (B), and represent means±SEM (n=6). $^+P<0.05$, compared to control. $^*P<0.05$, compared to neurotoxins.

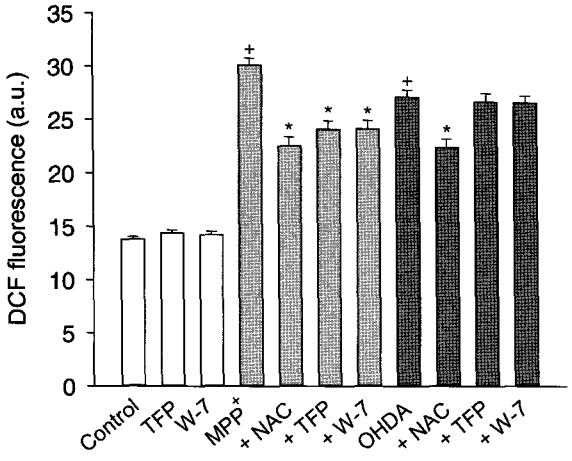

Fig. 5. Effect of trifluoperazine and W-7 on ROS formation due to MPP^+ or 6-OHDA. PC12 cells were treated either with neurotoxins in the presence of compounds (1 mM N-acetylcysteine [NAC], 1 μM trifluoperazine [TFP] and 0.5 μM W-7) or with calmodulin antagonist alone for 24 h. The values are expressed as arbitrary units (a.u.) of fluorescence for ROS formation. Data represent means±SEM (n=6). $^+P<0.05$, compared to control. $^*P<0.05$, compared to neurotoxins.

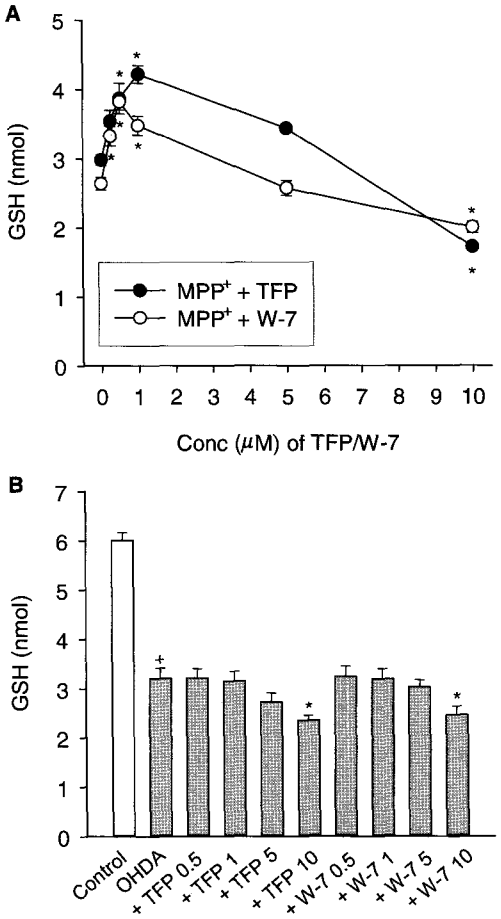

Fig. 6. Effect of trifluoperazine and W-7 on GSH depletion due to MPP^+ or 6-OHDA. PC12 cells were treated with neurotoxins in the presence of compounds (0.25~10 μM of trifluoperazine [TFP] and W-7) for 24 h. The values are expressed as nmol/mg protein for GSH contents. Data represent means±SEM (n=6). $^+P<0.05$, compared to control. $^*P<0.05$, compared to neurotoxins.

et al, 2000). The present study investigated the effect of trifluoperazine or W-7 against the change in GSH contents due to neurotoxins. The GSH content in the control PC12 cells was 5.98±0.19 nmol/mg protein. Treatment with MPP^+ or 6-OHDA for 24 h decreased the GSH contents by 50 and 47%, respectively. At the concentrations less than 5 μM, trifluoperazine and W-7 significantly inhibited the MPP^+-induced decrease in the GSH contents in PC12 cells (Fig. 6). The maximum inhibitory effect of antagonists on the MPP^+ cytotoxicity was achieved at 0.5~1 μM; beyond these concentrations the inhibitory effect declined. Meanwhile, both compounds at 5~10 μM did not show an inhibitory effect because of their cytotoxicity. In contrast to MPP^+, the 6-OHDA-induced depletion of GSH was not attenuated by 0.25~10 μM of trifluoperazine and W-7.

DISCUSSION

Neuronal cell deaths due to MPP^+ and 6-OHDA are mediated by opening of the mitochondrial permeability transition pore, leading to the release of cytochrome c and activation of caspases, and disturbance of intracellular Ca^{2+} homeostasis (Cassarino et al, 1999; Lotharius et al, 1999; Lee et al, 2002). The present results show that treatment with MPP^+ or 6-OHDA causes the release of cytochrome c into the cytosol due to mitochondrial dysfunction, leading to activation of caspase-3 in PC12 cells. However, it has been suggested that the cytotoxic effect of MPP^+ is mediated by the toxic actions, which are different from 6-OHDA (Lotharius et al, 1999; Lee et al, 2002). As previously mentioned, the inhibitor of the mitochondrial permeability transition CsA exhibited an inconsistent effect on the cytotoxicity of MPP^+ and 6-OHDA. The Ca^{2+}-induced mitochondrial swelling is not affected by the addition of 6-OHDA (Berman & Hastings, 1999). In addition, 6-OHDA induces cell death in chromaffin cells without intervention of mitochondrial swelling (Galindo et al, 2003). Trifluoperazine is well known as a calmodulin antagonist (Benaim & Villalobo, 2002) and can prevent formation of the mitochondrial permeability transition due to various insults (Lemasters et al, 1999). However, the effects of trifluoperazine and W-7 on the cytotoxicities of MPP^+ and 6-OHDA have not been clarified. At the concentrations, which did not induce a significant cytotoxicity, trifluoperazine and W-7 significantly attenuated the MPP^+-induced mitochondrial dysfunction and cell death in PC12 cells. The present findings suggest that trifluoperazine and W-7 reduce the cytotoxicity of MPP^+ by inhibition of the mitochondrial permeability transition and calmodulin. Meanwhile, the 6-OHDA-induced cell death seems to be mediated by the actions, which are different from MPP^+.

MPP^+ causes neuronal cell death by inducing mitochondrial dysfunction and increase in the formation of ROS (Cassarino et al, 1999; Lee et al, 2000; Obata, 2002). In contrast, the MPP^+-induced decrease in the [^{3}H] dopamine uptake in PC12 cells is not mediated by formation of ROS (Fonck & Baudry, 2001). The autoxidation of 6-OHDA

produces ROS and quinones, which induce apoptosis in human neuroblastoma cell line SH-SY5Y and rat PC12 cells (Kim et al, 2001; Jordán et al, 2004). Nevertheless, the involvement of hydrogen peroxide, a cell permeable oxidant, in the 6-OHDA-induced mitochondrial damage and cell death is not suggested in neuroblastoma (N-2A) cells (Mazzio et al, 2004). The inhibitory effect of antioxidants suggests that compared to MPP^+, the cytotoxic effect of 6-OHDA is chiefly mediated by ROS rather than nitrogen species. On the basis of the contradictory findings, we assessed whether the toxic effect of both neurotoxins is ascribed to the formation of ROS and the depletion of GSH. During the apoptotic process, drops in GSH levels and concomitant increases in ROS are detected (Tan et al, 1998; Chandra et al, 2000). This finding was also observed in the present study. Drops in the GSH contents are considered to increase the sensitivity of cells to toxic substances. The inhibitory effect of the free radical scavengers and the formation of ROS suggest that MPP^+ and 6-OHDA evoke mitochondrial damage in PC12 cells by inducing the formation of ROS and the decrease in the GSH contents. Trifluoperazine has been shown to reduce oxidative stress-induced cell death by suppressing the cytochrome c release and mitochondrial depolarization (Shen et al, 2001; Rodrigues et al, 2002). Trifluoperazine and chlorpromazine appear to protect rat heart myocardium H9c2 cells against the toxicity of rotenone by suppressing the formation of ROS due to depletion of ATP (Yaglom et al, 2003). The inhibitory effect of trifluoperazine and W-7 on MPP^+-induced cell death was approximately correlated with their effect on the GSH depletion. The results therefore suggest that trifluoperazine and W-7 may reduce the mitochondrial membrane permeability change due to MPP^+ in PC12 cells by suppressing increase in the oxidative stress. Meanwhile, it is unlikely that the 6-OHDA-induced mitochondrial dysfunction and increased oxidative stress are affected by inhibition of the mitochondrial permeability transition and by calmodulin inhibition.

In conclusion, the results suggest that trifluoperazine and W-7 may attenuate the cytotoxicity of MPP^+ by inhibition of the mitochondrial permeability transition and calmoduin. Meanwhile, the cytotoxic effect of 6-OHDA appears to be mediated by the actions, which are different from MPP^+.

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