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Protective effect of verbascoside on 1-methyl-4-phenylpyridinium ion-induced neurotoxicity in PC12 cells

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Abstract

The neuroprotective effects of verbascoside, one of phenylpropanoid glucoside isolated from the Chinese herbal medicine *Buddleja officinalis* Maxim, on 1-methyl-4-phenylpyridinium ion (MPP^+) induced apotosis and oxidative stress in PC12 neuronal cells were investigated. Treatment of PC12 cells with MPP^+ for 48 h induced apoptotic death as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry, the activation of caspase-3 measured by the caspase-3 activity assay kit, the reduction in mitochondrial membrane potential with laser scanning confocal microscopy and the increase in the extracellular hydrogen peroxide level. Simultaneous treatment with verbascoside markedly attenuated MPP^+-induced apoptotic death, increased extracellular hydrogen peroxide level, the activation of caspase-3 and the collapse of mitochondrial membrane potential. These results strongly indicate that verbascoside may provide a useful therapeutic strategy for the treatment of oxidative stress-induced neurodegenerative disease such as Parkinson's disease.

Keywords: *Buddleja officinalis* Maxim; Verbascoside; Phenylpropanoid glucoside; Oxidative stress; Apoptosis; Parkinson's disease

1. Introduction

The progressive neurodegeneration of nigrostriatal dopaminergic pathway is one of the principal factors accounting for the appearance of motor dysfunctions in Parkinson's disease. Even though the cause of Parkinson's disease remains answered, several lines of evidence strongly suggest involvement of oxidative stress, finally leading to neuronal death by the excessive generation of free radicals (Coyle and Puttfarcken, 1993; Gotz et al., 1994). Recently, attention has been focused on a wide array of natural antioxidants that are able to scavenge free radicals and protect cells from oxidative damage, such as verbascoside, a phenylpropanoid glucoside distributed in many medicinal plants (Deepak et al., 1999). Previous studies had found many phenylpropanoid glucosides including verbascoside showed a strong free radical scavenging activity (Li et al., 1999; Gao et al., 1999; Wang et al., 1996). In the present study, we have examined the possible inhibitory effect of verbascoside on neurotoxicity induced by 1-methyl-4-phenylpyridinium ion (MPP^+) in cultured PC12 cells that retain dopaminergic characteristics and have been widely used for neurobiological and neurochemical studies.

2. Material and methods

2.1. Materials

Verbascoside (Fig. 1) from *Buddleja officinalis* Maxim was kindly supplied by Dr. Mingju Liu (Department of Orthopaedics and Traumatology, The Chinese University of Hong Kong, Hong Kong, China). The purity of the compounds was more than 98% by high-performance liquid chromatography (HPLC) analysis. Dulbecco's modified Eagle's medium (DMEM), horse serum and fetal calf serum were purchased from Gibco BRL; poly-L-lysine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), MPP^+, horseradish peroxidase, 2,2'-azinobis(3-

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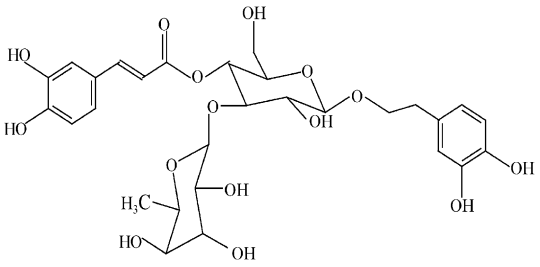

Fig. 1. Chemical structure of verbascoside.

ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), were purchased from Sigma (St. Louis, MO, USA). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was from Boehringer Mannheim, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1) and caspase-3 assay kit were obtained from Molecular Probes (Eugene, OR, USA). All other reagents or drugs were of analytical grade.

2.2. Cell culture and treatment

PC12 cells were maintained in DMEM, supplemented with 10% horse serum, 5% fetal calf serum and 2 mM L-glutamine, in a humified incubator aerated with 95% air and 5% CO_2 at 37 °C. All experiments were carried out 24–48 h after cells were seeded. The cells were routinely harvested by trypsinization 0.25% when the cells approached the subconfluent stage and were plated in 25-cm culture flasks split at 1:6.

2.3. Analysis of cell viability

The cell viability was determined using a modified MTT assay as described previously (Yamamoto et al., 2000). In brief, PC12 cells were seeded in 96-well plates at a density of 1×10^4 cells per well. The cultures were grown for 48 h, then the medium was changed to that containing various concentrations of verbascoside (0.1, 1 or 10 μg ml^{-1}) or 200 μM MPP^+. After incubation for up to 48 h, MTT solution (5 mg ml^{-1} in DMEM) was added to the 96-well plates and the cells were allowed to incubate for 4 h at 37 °C. After the medium had been removed, the cell and dye crystals were solubilized by adding 200 μl of dimethylsulfoxide (DMSO), and the absorption was measured at 570 nm (540 nm as a reference) with a model 550-microplate reader (Bio-Rad).

2.4. Determination of extracellular hydrogen peroxide

The level of extracellular hydrogen peroxide was determined by the horseradish peroxidase/ABTS method (Higuchi and Matsukawa, 1998), which is based on the horseradish peroxidase-catalyzed oxidation of ABTS. Cells cultured in phenol red-free DMEM were exposed to MPP^+ for 48 h with or without verbascoside. Then 0.4 ml of cell culture supernatant was mixed with 0.4 ml of reaction mixture (5 mM ABTS, 0.2 U horseradish peroxidase, 0.2 M acetate buffer, pH 4.0). After incubation at 37 °C for 20 min, the absorption at 420 nm was measured immediately. Hydrogen peroxide was used as an external standard.

2.5. Evaluation of apoptosis and necrosis

Both untreated and treated cells were harvested, washed and double-stained by using an Annexin V-FITC apoptosis detection kit. This kit is based on the observation that soon after initiating apoptosis most cell types translocate the membrane phospholipid phosphatidylserine from the inner-face of the plasma membrane to the cell surface. Annexin V has a strong affinity for phospholipid phosphatidylserine and therefore serves as a probe for detecting apoptosis. Cells that have lost membrane integrity will show red staining (propidium iodide) throughout the nucleus and therefore will be easily distinguishable from the apoptotic cells. Samples were incubated for 10 min in the dark with annexin V and propidium iodide. After 10 min incubation at room temperature in the dark, flow cytometry was performed with a FACScan (Becton Dickinson, Heidelberg, Germany). Annexin V-FITC and propidium iodide related fluorescence was recorded using FL1-H (525 nm) and FL2-H (575 nm) filters, respectively.

2.6. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential of control and treated PC12 cells was studied by using the probe JC-1. Cells were loaded by changing the culture medium to phosphate-buffered saline containing 1 μM JC-1 for 10 min at 37 °C. JC-1 is a metachromatic probe able to enter the mitochondria selectively (Cossarizza et al., 1995). The green fluorescent JC-1 exists as a monomer at low concentrations or at low membrane potential. However, at higher concentrations, JC-1 forms red fluorescent "J-aggregates". Both components of the dye are known to be sensitive to the mitochondrial membrane potential and changes in the ratio between green and red fluorescence can provide information regarding the mitochondrial membrane potential. The fluorescent dye was excited at 490 nm, and the fluorescence intensities of both monomer and aggregated JC-1 molecules were recorded at 590 nm with a confocal scanning laser microscope.

2.7. Measurement of caspase-3 activity

The activation of caspase-3 was determined using the caspase-3 activity assay kit, a fluorimetric immunosorbent enzyme assay that uses a monoclonal antibody to capture caspase-3 from cellular lysates, thus providing a greater specificity than similar commercial kits. Briefly, after exposure to MPP^+ with or without verbascoside treatment, cells ($>10^6$) were harvested and washed in phosphate-buffered

saline. Each cell sample or control was resuspended in cell lysis buffer comprising 10 mM Tris–HCl (pH 7.5), 130 mM NaCl, 1% Triton and 10 mM NaH_2PO_4. The lysed cells were centrifuged to pellet the cellular debris. Fifty microliters cell lysate was combined with the same volume of the reaction buffer consisting of 20 mM piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES, pH 7.4), 4 mM ethylene diamine tetraacetic acid (EDTA), 0.2% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) and 5 mM rhodamine110 bis-(*N*-CBZ–L-aspartyl–L-glutamyl–L-valyl–L-aspartic acid amide) (Z-DEVD-ER110), and was incubated at 37 °C for 30 min in the dark. The intensity of fluorescence of Z-DEVD-ER110 substrate was then measured in a fluorescence microplate reader using excitation at 485 ± 10 nm and emission detection at 535 ± 12.5 nm.

2.8. Statistical analysis of the data

The data are expressed as means ± S.E.M. Statistical comparisons were made by using Student's *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of verbascoside on MPP^+-induced cell death

PC12 cells were exposed to MPP^+ and cell viability was assessed by the MTT reduction assay. MPP^+ 200 μM killed 40–50% of the cells upon 48 h treatment and its cytotoxic effects were attenuated in the presence of verbascoside (0.1, 1 or 10 μg ml^{-1}) (Fig. 2). Verbascoside at these concentrations exhibited cytoprotective effects in a dose-dependent manner (35%, 46% and 59% of neuroprotection, respectively) and the compound alone did not cause any apparent cytotoxicity (data not shown).

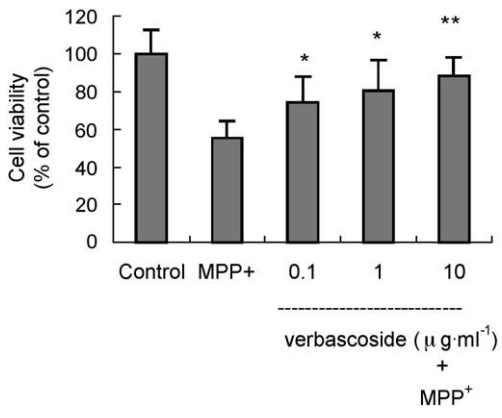

Fig. 2. Effects of verbascoside on the viability of MPP^+-treated PC12 cells. Cell viability was assessed by the MTT method as described in Materials and methods. Cells were treated with 200 μM MPP^+ for 48 h in the absence or presence of verbascoside. Data are expressed as percent of values in untreated control cultures, and are means ± S.D. of four experiments. * $P < 0.05$, ** $P < 0.01$ in comparison with cells exposed to 200 μM MPP^+.

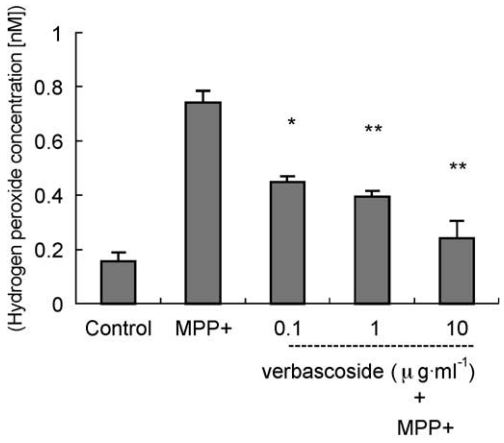

Fig. 3. Extracellular hydrogen peroxide accumulation in control PC12 cells and PC12 cells after 48 h exposure to 200 μM MPP^+ alone or associated with verbascoside. Data are means ± S.D. of four experiments. * $P < 0.05$, ** $P < 0.01$ in comparison with cells exposed to 200 μM MPP^+.

3.2. Effect of verbascoside on MPP^+-induced accumulation of extracellular hydrogen peroxide

Upon exposure to MPP^+ for 48 h, the extracellular hydrogen peroxide level increased significantly as shown in Fig. 3. The extracellular hydrogen peroxide level was 0.74 ± 0.04 nM 48 h after the addition of MPP^+, which was much higher than that of normal cells (0.16 ± 0.03 nM). Simultaneous treatment with verbascoside effectively prevented the accumulation of extracellular hydrogen peroxide, the level were reduced to 0.45 ± 0.02, 0.40 ± 0.02 and 0.24 ± 0.06 nM, respectively.

3.3. Effect of verbascoside on MPP^+-induced phospholipid phosphatidylserine exposure

Fig. 4 shows such a display of propidium iodide versus annexin-V fluorescence. The intensity values for classification of the cells in positive and negative classes were determined from histogram analyses of signals from propidium iodide only and annexin V-FITC only. The lower left quadrants of the cytograms show the viable cells, which exclude propidium iodide and are negative for annexin V-FITC binding. The lower right quadrants represent the apoptotic cells, annexin V-FITC positive and propidium iodide negative. The upper right quadrants contain necrotic and late apoptotic cells, positive for annexin V binding and for propidium iodide uptake. The upper left quadrants represent cells damaged during the procedure. 10 000 cells were analyzed under each condition. In the control, most neurons are healthy (90.9%). PC12 cells exposed to 200 μM MPP^+ revealed that of the annexin V positive cells (31.9%), 7.01% were propidium iodide negative (lower

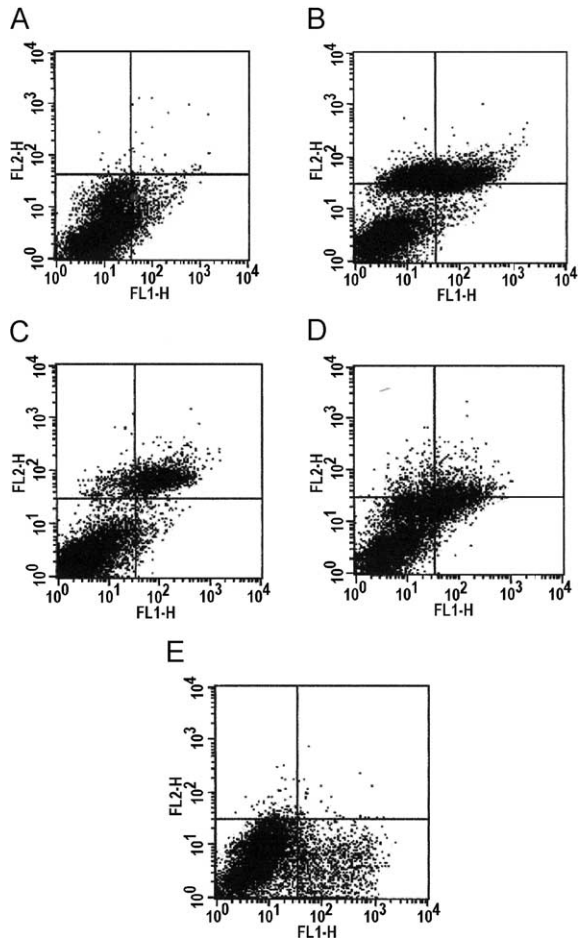

Fig. 4. Flow cytometric histograms of control PC12 cells and PC12 cells after 48 h exposure to 200 μM MPP^+ alone or associated with verbascoside (0.1, 1 and 10 μg ml^{-1}). After incubation, cells were harvested and labeled with a combination of annexin V-FITC and propidium iodide. (A) Control; (B) 200 μM MPP^+ treated alone; (C) 200 μM MPP^+ + 0.1 μg ml^{-1} verbascoside; (D) 200 μM MPP^+ + 1 μg ml^{-1} verbascoside; (E) 200 μM MPP^+ + 10 μg ml^{-1} verbascoside.

right quadrants), while 24.9% were propidium iodide positive (upper right quadrant), which indicated background early apoptosis and late apoptosis/necrosis, respectively (Fig. 4B). Simultaneous incubation with 0.1, 1 or 10 μg ml^{-1} verbascoside significantly reduces the number of cells labeled with annexin-V. The percentage of apoptosis/necrosis neurons was significantly decreased to 22.1%, 15.05% and 12.4%, respectively (Fig. 4C–E).

3.4. Effect of verbascoside on MPP+-induced reduction of mitochondrial membrane potential

To confirm that MPP^+ induced a mitochondrial membrane potential reduction in PC12 cells, confocal laser scanning microscopy was used to visualize their fluorescence dye-stained mitochondria. When cultured PC12 cells were loaded with JC-1 as described in Materials and methods, the monomeric JC-1 appears as a green fluorescence emission, while the J-aggregates of JC-1 were viewed red. Control PC12 cells (Fig. 5A) preferentially displayed red fluorescence (red/green ratio is 6.91 ± 0.17), indicating that a high fraction of mitochondria are in the energized state. However, decreases in mitochondrial energy transduction as indicated with the decrease of red/green ratios (0.37 ± 0.05) in PC12 cells were observed following 48 h of treatment of MPP^+, indicated by the disappearing red color and the swift appearance of the green fluorescence emission (Fig. 5B). Verbascoside attenuated the MPP^+-induced collapse of mitochondrial membrane potential in PC12 cells (Fig. 5C–E). There was a gradual recovery of the mitochondrial membrane potential in PC12 cells treatment simultaneously with verbascoside as indicated by the reappearance of red-stained mitochondria. The quantitative analysis of the red/green ratios also showed the dose-dependent increase of ratios (1.64 ± 0.09, 5.03 ± 0.05 and 5.07 ± 0.06, respectively).

3.5. Effect of verbascoside on MPP^+-induced activation of caspase-3

Caspases are the molecular machinery that drives apoptosis, and are responsible for the morphologic and biochemical characteristics of apoptotic cells. Following 48 h treatment of PC12 cells with 200 μM MPP^+, we detected a 125.19% increase of caspase-3-like activity compared with the control cells. In contrast, PC12 cells which were simultaneously treated with verbascoside (0.1, 1 or 10 μg ml^{-1}) showed a significant decrease in caspase-3 activity compared with the MPP^+-treated cells at the same time

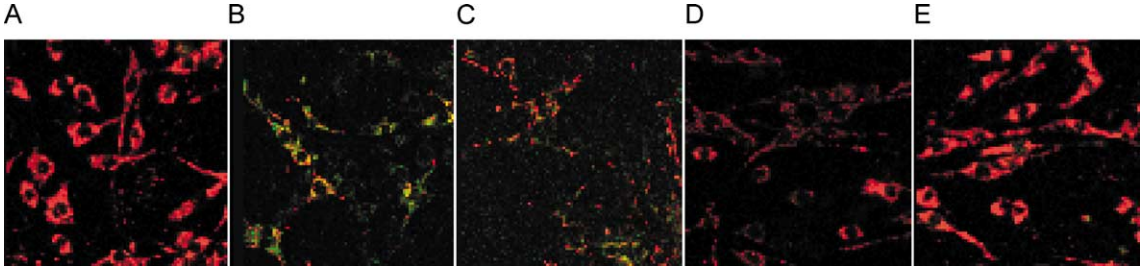

Fig. 5. Laser confocal scanning microscope images of JC-1 fluorescence (as a indictor of mitochondria membrane potential) in PC12 cells after 48 h exposure to 200 μM MPP^+ alone or associated with verbascoside (0.1, 1 and 10 μg ml^{-1}). (A) Control; (B) 200 μM MPP^+ treated alone; (C) 200 μM MPP^+ + 0.1 μg ml^{-1} verbascoside; (D) 200 μM MPP^+ + 1 μg ml^{-1} verbascoside; (E) 200 μM MPP^+ + 10 μg ml^{-1} verbascoside.

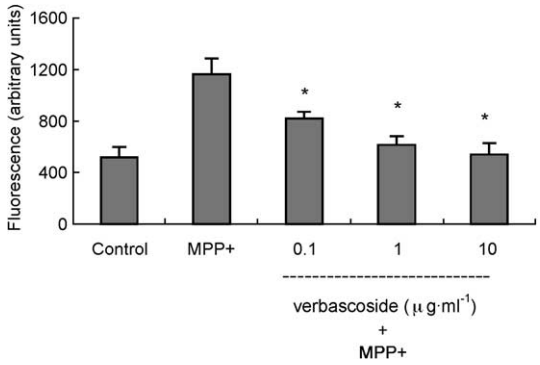

Fig. 6. Prevention of MPP^+-induced caspase-3 activation by verbascoside. Apoptosis in PC12 cells induced with MPP^+ involves caspase-3 activation. Cells were treated with 200 μM MPP^+ for 48 h in the absence or presence of verbascoside, then lysates were assayed for the cleavage of fluorogenic caspase substrates as detailed in Materials and methods. Values are means ± S.D. Data shown are from three independent experiments. $*P<0.05$, $**P<0.01$ in comparison with cells exposed to 200 μM MPP^+.

point (provided 29.6%, 47.2% and 53.7% protection, respectively) (Fig. 6). The results show that treatment with verbascoside resulted in the inhibition of MPP^+-induced activation of caspase-3.

4. Discussion

In our study, we first demonstrate that verbascoside, isolated from *B. officinalis* Maxim, has significant neuroprotective effects against MPP^+-induced apoptotic or necrotic death via mitochondria dysfunction in PC12 cells. The compound protected neuronal viability against MPP^+-induced toxicity in a dose-dependent manner. Further, we demonstrated that verbascoside also protected against the MPP^+-induced apoptosis/necrosis measured using flow cytometric analysis and caspase-3 activity assay. Moreover, treatment of PC12 cells with verbascoside significantly prevented the MPP^+-induced mitochondria dysfunction.

At present, the cellular and molecular mechanisms that underlie the action of verbascoside are not fully understood. However, our results demonstrate that several mechanisms, separately or in association, may be involved in the neuroprotective effects of verbascoside. The antioxidant effect is a possible mechanism for verbascoside-mediated neuroprotection.

It is well known that many types of chemical and physiological inducers of oxidative stress are able to cause apoptotic cell death (Slater et al., 1995; O'Brien et al., 2000). Reactive oxygen species produced by MPP^+ such as hydrogen peroxide is produced during the redox process (Rhee, 1999). Hydroxyl radical reacts rapidly with almost every cellular macromolecule, which can ultimately lead to apoptotic or necrotic cell death. Previous studies have found that dAMP radical anions and dGMP hydroxyl radical adducts can be repaired by verbascoside (Li et al., 1996; Shi et al., 1999). Several phenylpropanoid glycosides have also been recently reported to possess free radical scavenging properties and protect oxidative stress-induced toxic injuries. Thus, verbascoside may have direct scavenging effects against reactive oxygen species. Study of the molecular structure of verbascoside also indicates that it possesses a potent capacity for scavenging free radicals. The number of phenolic hydroxyl group in the structure of the verbascoside may be related to its scavenging activities.

Other mechanisms could also be pertinent. High levels of mitochondrial membrane potential are necessary to maintain closure of a multi-protein pore and the permeability transition pore (Tatton and Olanow, 1999). If the permeability transition pore opens, the proton gradient and mitochondrial membrane potential are lost and the concentrations of solutes smaller than 1.5 kDa equilibrate across the mitochondrial membranes resulting in mitochondrial swelling and fracture of the inner and outer mitochondrial membranes. The membrane fracture allows the escape of mitochondrial factors that induce apoptotic degradation (Susin et al., 1998). Caspase-3 activates DNA fragmentation factor, which in turn activates endonucleases, which cleave nuclear DNA. The fact that verbascoside inhibited the reduction of mitochondrial membrane potential induced by MPP^+ suggests that verbascoside may have the capacity to counteract the toxicity of MPP^+ by inhibiting the opening of mitochondrial permeability transition pore and dysfunction. Further studies of neuroprotective mechanisms of verbascoside in detail are necessary before definite conclusions can be drawn.

In summary, verbascoside has multifunctionally neuroprotective effects and may be useful for treatment of patients with Parkinson's disease.

Acknowledgements

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