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Neuroprotective effect of catalpol against MPP^+-induced oxidative stress in mesencephalic neurons

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Abstract

The neuroprotective effects of catalpol, an iridoid glycoside present in the roots of *Rehmannia glutinosa*, on 1-methyl-4-phenylpyridinium (MPP^+)-induced oxidative stress in cultured mesencephalic neurons, especially dopaminergic neurons, were investigated. Exposure of mesencephalic neurons to 10μM MPP^+ induced a leakage of lactate dehydrogenase (LDH) and decreased cell viability, measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Catalpol increased neuron viability and markedly attenuated MPP^+-induced dopaminergic neuron death in a dose-dependent manner. In order to clarify the neuroprotective mechanism of catalpol, mitochondrial function, the activities of endogenous antioxidants and the lipid peroxide content were measured. The results indicated that catalpol prevented the MPP^+-induced inhibition of complex I activity and the loss of mitochondrial membrane potential. In addition, catalpol reduced the content of lipid peroxide and increased the activity of glutathione peroxidase and superoxide dismutase. Taken together, the above results suggest that catalpol may be a candidate drug for the treatment of oxidative stress-induced neurodegenerative disease.

Keywords: Neuroprotective; Catalpol; Complex I; Oxidative stress; Mitochondria

1. Introduction

Parkinson's disease is an aged-related neurodegenerative disorder characterized by the loss of dopaminergic neurons in the nigrostriatal pathway. Although the mechanism still remains to be elucidated, the combination of mitochondrial dysfunction and increased oxidative stress is hypothesized to contribute to the selective degeneration of nigrostriatal dopaminergic neurons (Jenner and Olanow, 1996; Brown and Yamamoto, 2003; Orth and Schapira, 2002). The neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) is selectively toxic to nigrostriatal dopaminergic neurons and is widely used in the testing of many antiparkinsonian agents.

MPP^+ is a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an inducer of Parkinsonism in humans, monkeys and mice (Hallman et al., 1984; Langston et al., 1984). MPTP is a neurotoxin that selectively injures the nigrostriatal system. In the brain, MPTP is metabolized by monoamine oxidase B (MAO-B) to form the active neurotoxic metabolite MPP^+, which is then taken up into dopaminergic neurons via the dopaminergic transporter, and inhibits the multienzyme complex I of the mitochondrial electron-transport chain (Nicklas et al., 1985). Several in vivo and in vitro studies have found that MPP^+ exerts oxidative stress on cells. MPTP treatment produces an increase in brain hydroxyl radicals in mice (Cassarino et al., 1997), and high concentrations of MPP^+ have been shown to increase reactive oxygen species in neuroblastoma cells (Cassarino et al., 1997). In animals, overexpression of antioxidant enzymes protects against MPTP toxicity (Przedborski et al., 1992), and antioxidant molecules protect against MPP^+ toxicity in neuronal cell lines and dopaminergic neurons in primary culture (Akaneya et al., 1995).

The involvement of mitochondrial dysfunction in Parkinson's disease is based on the finding that the activity of complex I of the electron-transport chain is significantly decreased in observed in the substantia nigra of postmortem brain (Schapira et al., 1990). The impairment of mitochondrial activity contributes to both reactive oxygen species generation and nigral cell loss. Excessive production of reactive oxygen species, such as

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superoxide anion, hydroxyl radical and hydrogen peroxide, may either directly damage the cellular macromolecule to cause cell necrosis or indirectly affect normal cellular signaling pathways and gene regulation to induce apoptosis (Facchinetti et al., 1998; Sugawara and Chan, 2003). Malondialdehyde, a stable metabolite of the free radical-mediated lipid peroxidation cascade, is widely used as a marker of oxidative stress. Studies have shown that biological systems have evolved with endogenous defense mechanisms to help protect against reactive oxygen species-induced cell damage. Superoxide dismutase (SOD) and glutathione peroxidase are endogenous antioxidant enzymes which play pivotal roles in preventing cellular damage caused by reactive oxygen species (Barlow et al., 2005; Husain et al., 2005).

Catalpol, an iridiod glucoside (Fig. 1) isolated from the roots of *Rehmannia glutinosa*, has been reported to induce neuronal differentiation in PC12 cells through activation of the intracellular signal transduction pathway (Yamazaki et al., 1996) and to attenuate apoptosis induced by H_2O_2 in PC12 cells in vitro (Jiang et al., 2004). The antioxidant property of catalpol is also well documented (Li et al., 2004). In addition, mitochondria are involved as producers of reactive oxygen species. Oxidative stress at the level of the mitochondria will result in the failure of enzymatic, transport and receptor systems (Ozawa, 1995). Moreover, mitochondrial dysfunction and oxidative stress are thought to play a role in the etiology of Parkinson's disease. Owing to the lack of evidence explaining the effects of catalpol on mitochondrial activity and oxidative stress, this study sought to determine whether catalpol could protect dopaminergic neurons from toxicity induced in MPP^+. Furthermore, the effects of catalpol on complex activity and antioxidative enzymes in MPP^+-treated mesencephalic neuron-enriched cultures were investigated in order to elucidate the neuroprotective mechanism.

2. Materials and methods

2.1. Materials

Catalpol was of analytical grade (purity>98%) and was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and dissolved in physiological saline. MPP^+, antimycin A, coenzyme Q1, and NADH were purchased from Sigma. The monoclonal anti-tyrosine hydroxylase (TH) antibody was purchased from Chemicon. SABC compound kits were from Sino-American Biotechnology Company. 2′, 7′-Dichlorofluorescein diacetate was obtained from Beyotime. Tissue culture media and fetal bovine serum were obtained from Gibco.

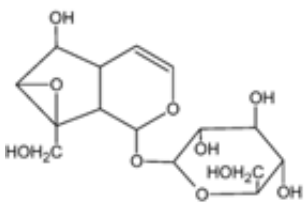

Fig. 1. The chemical structure of catalpol.

2.2. Primary mesencephalic neuron-enriched cultures and treatment

Mesencephalic neuron-enriched cultures were prepared from the ventral mesencephalic tissues of embryonic day 13/14 mice as described previously (Gao et al., 2003; Qin et al., 2004). Briefly, dissociated cells were seeded at 5×10^5/well and 6×10^6 onto poly-D-lysine-coated 24-well plates and 75-cm^2 T-flask. Cells were maintained at 37°C in a humidified atmosphere of 5 % CO_2 and 95 % air in Dulbecco's modified Eagle's medium/nutrient F12 (DMEM/F12) containing 10% fetal bovine serum (FBS), 50 U/ml penicillin, 1.2 g/l sodium bicarbonate and 2 mM L-glutamine. Glial proliferation was suppressed by the inclusion of cytosine β-D-arabinofuranoside (Ara-C, 10 μM) at 48 h. Two days later, the β-D-arabinofuranoside-containing medium was replaced with fresh complete medium. Seven-day-old cultures were used. Immunocytochemical analysis indicated that the purity of neurons was ≥95% The cells were cultured in the presence or absence of 10μM MPP^+ for 48 h. When the effects of catalpol on cells were studied, the various concentrations of catalpol were added for 30 min prior MPP^+ treatment. Thirty minutes later, MPP^+ and different concentrations of catalpol were added and incubated for 48 h in growth media.

2.3. Analysis of cell viability

After the above cell treatment protocol, the level of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasolium bromide (MTT) was quantified as previously described (Mosmann, 1983; Vian et al., 1995). Briefly, cells in 96-well plates were rinsed with phosphate-buffered saline, and MTT (0.5 mg/ml) was added to each well. The microplate was incubated at 37 °C for 3 h. At the end of the incubation period, the medium with MTT was removed and 200 μl dimethyl sulfoxide (DMSO) was added to each well. The plate was shaken on a microplate shaker to dissolve the blue MTT-formazan. Absorbance was read at 570 nm on a microplate reader. Cell viability is expressed as a percentage of that of the control culture.

2.4. Immunocytochemistry

Immunostaining was performed as previously described (Zhou et al., 2005). Dopaminergic (DA) neurons were recognized with a rat monoclonal anti-TH antibody (1:300, Chemicon). Briefly, formaldehyde-fixed cultures were treated with 1% hydrogen peroxide followed by sequential incubation with blocking solution for 30 min. Cells were incubated overnight at 4 °C or 37 °C for 2 h with primary anti-TH diluted in antibody diluent. The bound primary anti-TH antibody was visualized after incubation with biotinylated secondary antibody, followed by the ABC reagents and color was developed with 3, 3′-diaminobenzidine. For morphological analysis, the images were recorded with an inverted microscope (OLYMPUS CK40) connected to a camera.

2.5. Isolation of mitochondria

The mitochondrial fraction was prepared as previously described (Menzies et al., 2002). Cells were washed in phosphate-buffered saline at the end of the treatment period, homogenized on ice in 10 volumes of 250 mM sucrose with 0.1 mM EGTA and 2 mM HEPES, pH 7.4, and the homogenates were centrifuged at 500 ×*g* for 5 min at 4 °C. The mitochondrial pellet and cytosolic fraction were obtained by centrifugation of the supernatant at 12,000 ×*g* for 10 min. The mitochondrial pellet was resuspended in sucrose medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, and 5 mM HEPES, pH 7.4, at a concentration of 2 μg protein/μl, and used for the measurement of complex I activity.

2.6. Measurement of complex I activity

Complex I activity was determined by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADH (Helmerhorst et al., 2002; Schapira et al., 1990). The reaction mixture contained 250 mM sucrose, 1 mM EDTA, 50 mM Tris–HCl, pH 7.4, 2 μg/ml antimycin A, 2 mM KCN, 0.15 mM coenzyme Q1, and 20–40 μg mitochondrial homogenate. The total assay volume was 1 ml and the reagents were pre-warmed for 2 min at 30 °C. The reaction was initiated by addition of 0.1 mM NADH and the rate of decrease in absorbance was monitored spectrophotometrically at 340 nm for 3 min. Rotenone (10 μg/ml) was used to inhibit complex I activity. Absorbance was monitored for the indicated time period before and after addition of rotenone, using a microplate spectro-photometer (JASCO, V-560).

2.7. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was monitored using the fluorescent dye Rhodamine 123 (Rh 123), a cell-permeable cationic dye, which preferentially partitions into mitochondria because of the highly negative mitochondrial membrane potential. Depolarization of the mitochondrial membrane potential results in the loss of Rh 123 from the mitochondria and a decrease in intracellular fluorescence (Satoh et al., 1997). Rh 123 was added to cultures to attain a final concentration of 10 μM for 30 min at 37 °C after cells were treated and washed with phosphate-buffered saline. The cells were collected and washed twice with phosphate-buffered saline. Fluorescence was read at 480 nm for excitation and 530 nm for emission with a fluorescence plate reader. (Genios, TECAN).

2.8. Biochemical assays

The activities of lactate dehydrogenase (LDH), SOD, glutathione peroxidase as well as the concentration of the malondialdehyde in the supernatant were all determined by using commercially available kits (Jiancheng Bioengineering), according to the manufacturer's instructions. After treatment with catalpol (0.05–0.5 mM) and MPP^+ (10 μM), the culture supernatants were collected for measuring enzyme activity. LDH leakage was calculated as the percentage of LDH in the medium versus total LDH activity in the cells. The assay of SOD activity was based on its ability to inhibit the oxidation of oxymine by O_2^- produced from the xanthine–xanthinoxidase system. One unit of SOD activity was defined as the amount that reduced the absorbance at 550nm by 50%. Glutathione peroxidase activity was assayed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H_2O_2 catalyzed by glutathione peroxidase. One unit of glutathione peroxidase was defined as the amount that reduced the level of GSH by 1 μmol L^{-1}. Lipid peroxidation was assessed by measuring the concentration of malondialdehyde, which can be measured at a wavelength of 532nm, formed by reaction with thiobarbituric acid.

2.9. Statistical analysis

Data are expressed as the means±S.E.M. Statistical evaluation of the data was performed by ANOVA. All estimates were conducted in triplicate. A value of p less than 0.05 was considered statistically significant.

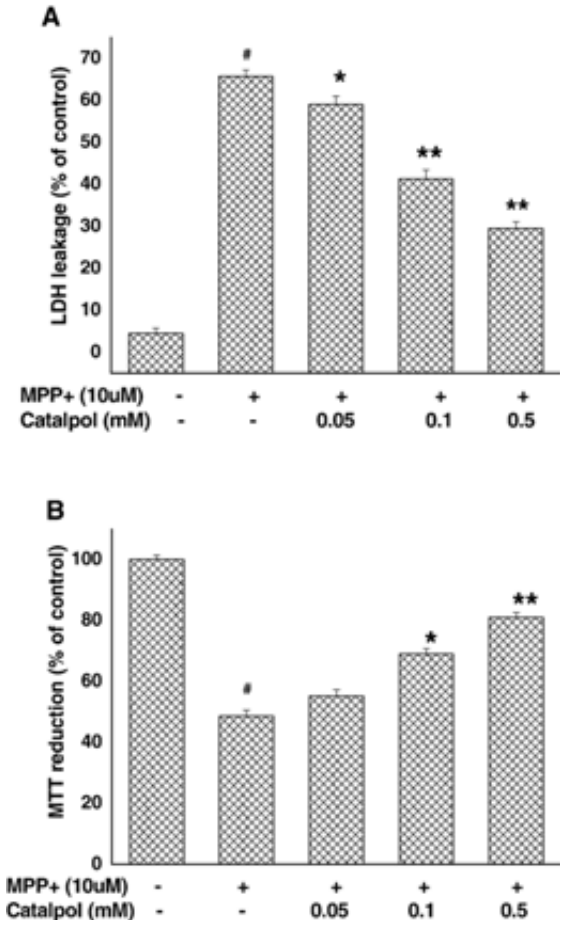

Fig. 2. Effects of catalpol on MPP^+-induced cell damage. Mesencephalic neurons were treated with 10 μM MPP^+ in the absence or presence of catalpol. LDH leakage (A) and viability of the cells (B) were determined after 48 h. Data are expressed as percent of values in untreated control cultures and are means±S.E.M. of three experiments. $^{\#}P<0.05$ in comparison with control, $^{*}P<0.05$ and $^{**}P<0.01$ in comparison with cells exposed to MPP^+ alone.

3. Results

3.1. Effects of catalpol on MPP^+-induced neuron damage

The effect of catalpol on cell viability was evaluated by MTT assay. The maximum concentrations of catalpol that did not affect cell viability was determined (0.5mM) before examining protective effect on neurons treated with MPP^+. The results in Fig. 2A showed that pretreatment of mesencephalic neurons with catalpol dose-dependently increased cell viability, and these findings were further verified by LDH assay (Fig. 2B). Thus, catalpol is effective in protecting mesencephalic neurons.

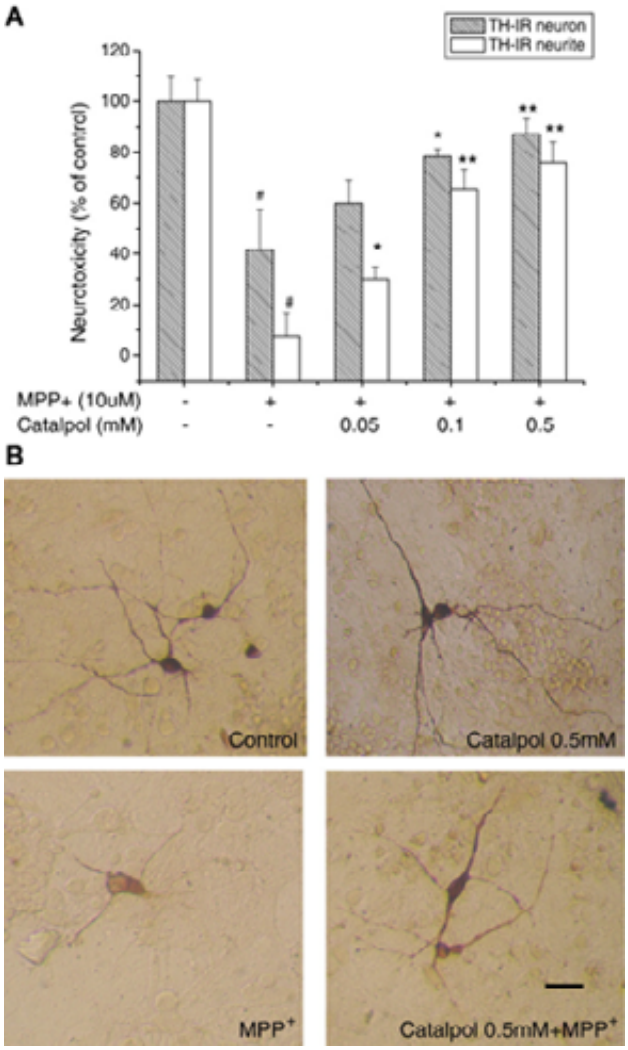

Fig. 3. Neuroprotective effects of catalpol against MPP^+-induced neurotoxicity. Mesencephalic neuron-enriched cultures were pretreated for 30 min with vehicle or the indicated concentrations of catalpol prior to treatment for 48 h with MPP^+(10 μM). After immunostaining, the number of TH-positive neurons and the average length of TH-positive neurites were quantified as described in the Materials and methods (A). Results are means±S.E.M from three independent experiments. $^{\#}P<0.05$ in comparison with control, $^{*}P<0.05$ and $^{**}P<0.01$ in comparison with cells exposed to MPP^+ alone. After immunostaining, the images were recorded with an inverted microscope connected to a camera (B). Healthy TH-positive neurons in the control cultures had extensive neurites and the MPP^+-induced loss of cell bodies and neuronal processes was reversed by catalpol pretreatment. Scale bar, 25 μm.

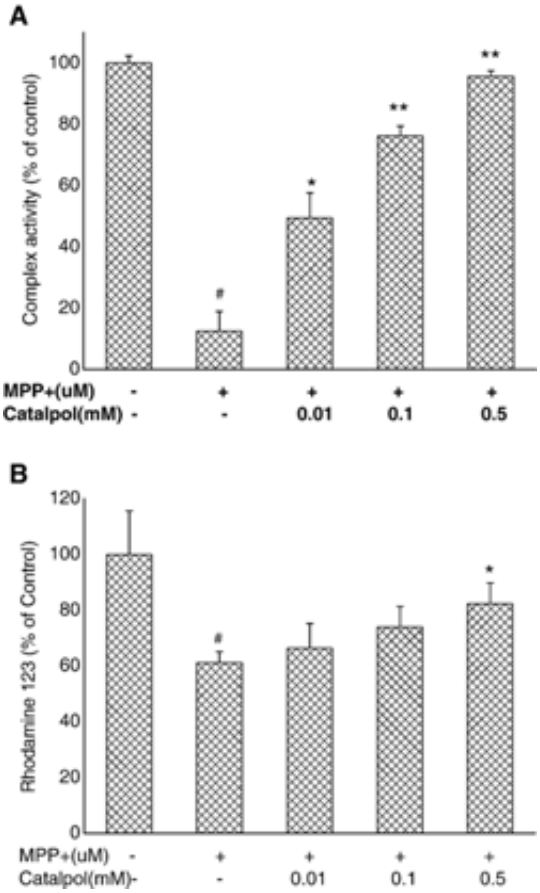

Fig. 4. Effects of catalpol on mitochondrial complex I activity and the loss of mitochondrial membrane potential in MPP^+-treated mesencephalic neuron-enriched cultures. Neuron-enriched cultures were pretreated with different concentrations of catalpol for 30 min prior to exposure to MPP^+. A. The activity of complex I. B. Mitochondrial membrane potential was measured with Rh 123 as described in Materials and methods. Results are expressed as mean±S.E.M of three experiments performed in triplicate. $^{\#}P<0.05$ in comparison with control, $^{*}P<0.05$ and $^{**}P<0.01$ in comparison with cells exposed to MPP^+ alone.

3.2. Catalpol protects dopaminergic neurons against MPP^+-induced neurotoxicity in mesencephalic neuron-enriched cultures

Mesencephalic neuron-enriched cultures were used to evaluated the effect of catalpol on MPP^+-induced dopaminergic neurodegeneration. Neuron-enriched cultures were pretreated with the desired concentrations of catalpol for 30min, then were treated with 10μM MPP^+ for an additional 48h. The extent of the degeneration of dopaminergic neurons was assessed by counting the number of TH-positive neurons and by morphological inspection. Immunocytochemical analysis of TH-positive neurons demonstrated that MPP^+ induced a significant decrease in the number of dopaminergic neurons. Pretreatment with catalpol (0.05–0.5mM) effectively reduced the degeneration of TH-positive cell bodies. Morphological inspection revealed that MPP^+ treatment not only decreased the number of TH-positive neurons, but also had an apparent inhibitory effect on the outgrowth of neurites. These characteristics were reversed by catalpol in a dose-dependent manner. Treatment with catalpol (0.5mM) alone had no effect on the morphology or number of TH-positive neurons (Fig. 3).

3.3. Effects of catalpol on mitochondrial function

After incubation of cells with 10 μM MPP^+ for 48 h, the mitochondrial membrane potential decreased to 61.1±3.97% of control. Pretreatment with low concentrations of catalpol failed to significantly ($P<0.05$) affect the membrane potential (Fig. 4B). However, a higher concentration of catalpol (0.5 mM) protected cells against the MPP^+-induced lowering of mitochondrial membrane potential (82.3±7.45 %). The activity of complex I in the mitochondrial fraction was measured spectrophotometrically as described in Materials and methods. As shown in Fig. 4A, 10 μM MPP^+ caused a significant decrease in complex I activity and pretreatment with catalpol blocked the effect of MPP^+. These characteristics were reversed by catalpol in a dose-dependent manner.

3.4. Effects of catalpol on the content of lipid peroxide in MPP^+-treated mesencephalic neurons

The selective vulnerability of the brain to oxidative damage arises from the low level of activity of antioxidant enzymes and the high content of endogenous heavy metals. Moreover, the abundance of polyunsaturated fatty acids in nerve cell membranes could lead to lipid peroxidation and impairment of membrane function. To evaluate whether the protective effect of catalpol was due to its direct inhibition of oxidative stress in mesencephalic neurons, the content of lipid peroxide was measured as described in Materials and methods. The results showed that pretreatment with catalpol dose-dependently decreased the MPP^+-induced the level of malondialdehyde (Fig. 5).

3.5. Effects of catalpol on the activities of SOD and glutathione peroxidase

As shown in Fig. 6A and B, MPP^+ treatment induced a significant decrease in the activities of superoxide dismutase and glutathione peroxidase. Pretreatment with catalpol (0.05–0.5 mM) significantly and dose-dependently increased the activities of SOD and glutathione peroxidase.

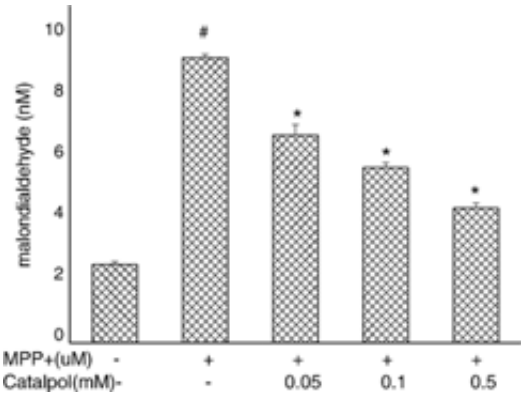

Fig. 5. Effects of catalpol on MPP^+-induced generation of malondialdehyde. Mesencephalic neuron-enriched cultures were pretreated for 30 min with indicated concentrations of catalpol prior to stimulation with MPP^+. Production of malondialdehyde was measured at a wavelength of 532 nm by reacting with thiobarbituric acid to form a stable chromophoric production as described in the Materials and methods. Results are means±S.E.M of three independent experiment. $^{\#}P<0.05$ in comparison with control, $^{*}P<0.05$ in comparison with cells exposed to MPP^+ alone.

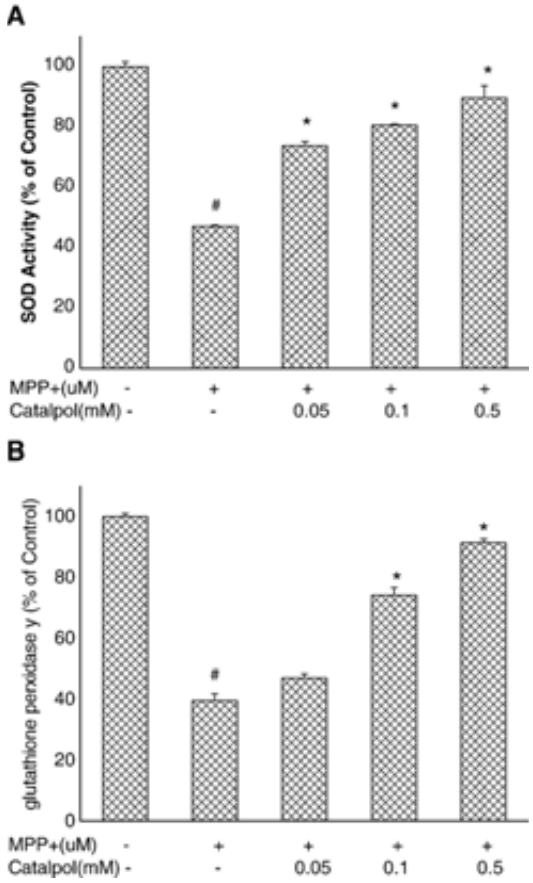

Fig. 6. Effects of catalpol on activities of antioxidant enzymes. Neuron-enriched cultures were pretreated with indicated concentrations of catalpol for 30 min prior to treatment with MPP^+. After 48 h, the effect of catalpol on activities of antioxidant enzymes was determined by detecting SOD (A) and glutathione peroxidase (B) released into supernatant. Results are means±S.E.M at least three independent experiments. $^{\#}P<0.05$ in comparison with control, $^{*}P<0.05$ in comparison with cells exposed to MPP^+ alone.

4. Discussion

The generation of free radicals is considered to be a major factor in the pathogenesis and progression of Parkinson's disease (Chiueh et al., 1994). The use of antioxidants has been reported to protect dopaminergic neurons against MPP^+-induced neurodegeneration (Le, 1994). Oxidative stress is a harmful condition that occurs when there is an excess of reactive oxygen species and/or a decrease in antioxidant levels. Therefore, removal of excess reactive oxygen species or suppression of their generation by antioxidants may be effective in preventing oxidative cell death. Recently, researchers have made considerable effort to search for natural substances with neuroprotective potential, and attention has been focused on a wide array of antioxidants that can scavenge free radicals and protect cells from oxidative damage. In a previous study, catalpol (0.65 mM) was found to protect hippocampal CA1 cells in vivo when

applied after severe global ischemia (Li et al., 2004). The involvement of catalpol in neuroprotection is supported further by the in vitro experiment that showed that concentrations ≤ 0.5 mM exerted neuroprotective effects in a dose-dependent manner. In contrast, 1 mM catalpol was cytotoxic per se. To evaluate catalpol as neuroprotective agent, we investigated the protective role of catalpol, using the MTT assay and LDH leakage measurements. The results demonstrate that MPP^+ induced cell death via oxidative stress in cultured mesencephalic neurons, and especially in dopaminergic neurons. However, the cytotoxic effects could be attenuated markedly by pretreatment with catalpol.

Findings from recent investigations indicate that the mitochondria play a prominent role in neuron death. Complex I is thought to be one of the major targets of both environmental and endogenous oxidative stressors in causing mitochondrial dysfunction in the nigra neurons of Parkinson's disease patients (Schapira, 2001). The role of mitochondrial dysfunction in the pathogenesis of Parkinson's disease is also supported by the fact that the dopaminergic toxin MPTP inhibits complex I (Benecke et al., 1993). The mitochondrial inhibition by MPP^+ leads to a decrease in cellular ATP levels and to the loss of mitochondrial membrane potential. In the present study, we observed that MPP^+ inhibits complex I activity and resulted in the loss of mitochondrial membrane potential. In contrast, pretreatment with catalpol significantly reduced the characteristic mitochondrial dysfunction.

Impairment of mitochondrial activity can also contribute to reactive oxygen species generation and result in nigral cell loss. Reactive oxygen species are important in causing neuron death. Reactive oxygen species, including superoxide anions, hydroxyl radicals, lipid hydroperoxides and their byproducts, are toxic to neurons by inducing lipid peroxidation, DNA fragmentation, and protein oxidation (Farber, 1994). These reactive oxygen species can be scavenged by endogenous antioxidants including SOD and glutathione peroxidase. The protective effect of catalpol against MPP^+-induced mesencephalic neuron damage was associated with increased levels of antioxidants: malondialdehyde, SOD and glutathione peroxidase are major endogenous antioxidants in brain. Reduction of the endogenous antioxidant content enhances oxidative stress and eventually results in cell death (Li et al., 2004; Ibi et al., 1999). Malondialdehyde is a byproduct of lipid peroxidation induced by free radicals and is widely used as a biomarker of oxidative stress. In the present study, the levels of SOD and glutathione peroxidase were reduced in the neurons due to MPP^+ but were significantly elevated by catalpol pretreatment. Moreover, catalpol decreased the contents of malondialdehyde in mesencephalic neurons exposed to MPP^+. These results suggest that catalpol is very effective in preventing oxidative stress triggered by deterioration of cellular functions, reducing the levels of reactive oxygen species.

This is the first report to demonstrate the protective effect of catalpol on mesencephalic neurons. The results suggest that catalpol can effectively attenuate MPP^+-induced dopaminergic neurotoxicity. Moreover, catalpol not only serves as free a radical scavenger but also prevents mitochondrial dysfunction and subsequent lipid peroxidation. Therefore, catalpol may be a candidate chemical for the treatment of oxidative stress-induced neurodegenerative disease.

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