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Catalpol protects mesencephalic neurons against MPTP induced neurotoxicity via attenuation of mitochondrial dysfunction and MAO-B activity

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ABSTRACT

Catalpol, an iridoid glucoside, separated from the root of *Rehmannia glutinosa Libosch*, has been known to show various neuroprotective effects. In humans and rodents, MPTP is well known to produce clinical, biochemical and neurochemical changes similar to those which occur in Parkinson's disease (PD). Furthermore, the accumulated evidence suggests that MPP^+, conversed by monoamine oxidase type B (MAO-B) in astrocytes principally, is the active metabolite of MPTP and the major cause to PD associated with mitochondrial dysfunction. In this study, we treated mesencephalic neuron-astrocyte and astrocytes cultures with MPTP (0.05 mM) respectively to investigate the neuroprotective effects of catalpol and the underlying protective mechanisms. Our results showed that pre-treatment with catalpol (0.5 mM) for 1 h prior to MPTP treatment attenuated mitochondrial dysfunction not only by reversing the activity of mitochondrial complex I, mitochondrial membrane potential (MMP), intracellular Ca^{2+} level, and ROS accumulation as well as mitochondrial permeability transition (MPT) pore opening in mesencephalic neuron-astrocyte cultures, but also inhibiting MAO-B activity to protect neurons from more MPP^+ toxicity produced in astrocytes. Together, all of these indicated that catalpol possesses potent neuroprotective activity and may be a potential anti-PD drug worthy for further study.

1. Introduction

Parkinson's disease (PD) is characterized by a loss of ventral midbrain dopaminergic (DA) neurons, which can be modeled by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Dauer and Przedborski, 2003). Nowadays, there is a growing body of evidence suggests that mitochondrial dysfunction involved in various neurodegenerative diseases including PD. Moreover, 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of MPTP, is the major cause to PD associated with mitochondrial dysfunction (Schapira, 1999).

Current evidence found that the toxic effect of MPTP is based on its conversion to MPP^+ by monoamine oxidase type B (MAO-B). This metabolism of MPTP has been shown to be localized principally in astrocytes, in which MAO-B is predominantly found (Ben-Shlomo and Bhatia, 2004). Previous evidence also illuminated that level of MPP^+ in astrocyte cultures was in the range known to be damaging to neurons following a 1-day exposure to MPTP, with level of 50 μM being sufficient to produce toxicity (Yang et al., 1988). Once in these cells, MAO-B rapidly converts the MPTP protoxin to the toxic MPP^+ at a 1:1 ratio (Berlin et al., 2000). Administration of MPTP to mice lacking MAO-B showed that SNpc neurons did not die (Shih and Chen, 1999) further demonstrating that MAO-B conversion of MPTP to MPP^+ is necessary to cause cell death.

After the conversion of MPTP in astrocytes, MPP^+ is selectively taken up by the high affinity dopamine uptake system and is subsequently accumulated within the mitochondria of DA neurons. There it disrupts oxidative phosphorylation by inhibiting complex I of the electron transport chain (ETC) (Smeyne and Jackson-Lewis, 2005). This damage can lead to a number of deleterious effects on mitochondrial function. These include impaired intracellular calcium (Ca^{2+}) buffering, the loss of mitochondrial membrane potential (MMP) as well as generation of reactive oxygen species (ROS) and inhibition of mitochondrial ATP production (Keeney et al.,

Abbreviations: CNS, central nervous system; DA, dopaminergic; DCFH, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorosecin diacetate; DMEM/F12, Dulbecco's modified Eagle's medium/nutrient F12; DMSO, dimethyl sulfoxide; ETC, electron transport chain; FBS, fetal bovine serum; Fura-2 AM, Fura-2 acetoxymethyl ester; GFAP, glial fibrillary acid protein; LDH, lactate dehydrogenase; MAO-B, monoamine oxidase type B; MMP, mitochondrial membrane potential; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPT pore, mitochondrial permeability transition pore; MTT, 3-[4,5-dimethylthiazol-2]-2,5 diphenyltetrazolium bromide; PBS, phosphate buffered saline; PD, Parkinson's disease; Rh123, rhodamine 123; ROS, reactive oxygen species; TH, tyrosine hydroxylase.

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2006). Simultaneously, associated with the inhibition of complex I was a release of cytochrome c from the mitochondrial membrane (Darios et al., 2003). In addition, several researches have shown the opening of the high-conductance mitochondrial permeability transition (MPT) pore induced by MPP^+ will result in triggering of an apoptotic cascade (Armstrong, 2006).

Catalpol, an iridiod glucoside, isolated from the traditional Chinese herbal medicines *Rehmannia glutinosa*, has been reported to attenuate apoptosis induced by H_2O_2 in PC12 cells (Jiang et al., 2004) and protecting DA neurons from LPS-induced neurotoxicity in mesencephalic neuron-glia cultures (Tian et al., 2006). In our laboratory, we also investigated the potential usefulness of catalpol in animal model of PD induced by rotenone (Mao et al., 2007).

Our previous data from Tian and Bi (Tian et al., 2007; Bi et al., 2008) have showed that when concentrations $\leqslant$0.5 mM catalpol exerted neuroprotective effects in a dose-dependent manner. Furthermore, some experiments in vivo also found that catalpol could enhance cognitive performance and protect mice brain from oxidative damage when injected at the does of 5 mg/kg or 10 mg/kg subcutaneously (Zhang et al., 2007). By far, no information is available concerning the neuroprotection of catalpol on mesencephalic neuron-astrocyte cultures damaged by MPTP. In the study reported here, we treated mesencephalic neuron-astrocyte and astrocytes cultures with MPTP (0.05 mM) to elucidate the neuroprotective effect of catalpol and the possible neuroprotective mechanisms in neurons related to astrocytes.

2. Materials and methods

2.1. Materials

Catalpol was separated from traditional Chinese herbal medicines *Rehmannia glutinosa* (Zhang et al., 2007) and diluted in phosphate buffered saline (PBS) for treatment. The monoclonal anti-tyrosine hydroxylase (TH) and anti-glial fibrillary acid protein (GFAP) antibodies were purchased from Chemicon. 2′,7′-dichlorofluorosecin diacetate (DCFH-DA) and Fura-2 acetoxymethyl ester (Fura-2 AM) were both purchased from Beyotime Institution of Biotechnology. MPTP was purchased from Sigma. Tissue culture media and fetal bovine serum were obtained from Gibco.

2.2. Primary astrocyte cell cultures

Astrocyte-enriched cultures were prepared from the midbrain of 1-day-old neonatal mice. Briefly, the meninges were removed from dissected midbrain. A single-cell suspension was obtained by mechanical dissociation. Then dissociated 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). Microglia and other non-adherent cells were removed by mild shaking for about 6 h at 180 rpm. The remaining cells were trypsinized and replated in culture plates at a density of 2×10^5 cells/cm^2, after 2–3 days were employed. Astrocytes were identified by immunocytochemical staining with anti-GFAP and the percentage of astrocytes was higher than 95%.

2.3. Primary mesencephalic neuron-astrocyte cocultures

Mesencephalic neurons were prepared from the ventral mesencephalic tissues of embryonic day 13/14 mice as described previously (Tian et al., 2007). Identification of midbrain DA neurons was performed using a monoclonal anti-TH. Then cocultures were established by plating mesencephalic neurons (1.25×10^5 cells/cm^2) on a confluent monolayer of astrocytes (2×10^5 cells/cm^2) grown in culture plates as described above. Cocultures were maintained in neuronal culture medium (DMEM/F12 containing 10% FBS). Seven-day-old cultures were used.

2.4. Cell treatment

The cells were divided into four groups: (1) control group: the cells were treated with PBS alone; (2) MPTP-treated group: the cells were treated with 0.05 mM MPTP (final concentration) alone for 24 h; (3) catalpol/MPTP-treated group: the cells were pretreated with 0.5 mM catalpol (final concentration) 1 h and then MPTP was added for 24 h; (4) catalpol-treated group: cells were pretreated with 0.5 mM catalpol for 1 h and then in the same manner with PBS instead of MPTP for 24 h.

2.5. Assessment of cell injury and cell viability

The release of cytosolic enzyme lactate dehydrogenase (LDH), an indicator of cytotoxicity, reflected a loss of membrane integrity in dying cells and was determined by a colorimetric assay. After cell treatment, the supernatants were collected for LDH measurement in the cell free medium. The cells remaining in each well were lysed in 0.5 ml lysis buffer (0.5% Triton X-100 in PBS). The release of intracellular LDH to the extracellular medium was measured at 340 nm using a microplate reader and was calculated as the percentage of LDH in the medium versus total LDH activity in the cells.

The viability was analyzed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Hansen et al., 1989). After treatment, MTT (5 mg/ml) was added to the cultures and incubated at 37 °C for an additional 3 h. Then supernatants were removed and 100 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formed blue formazan. Absorbance was read at 570 nm on a microplate reader. Cell viability was expressed as a percentage of the control culture value.

2.6. Isolation of mitochondria

The mitochondrial fraction was prepared as previously described (Menzies et al., 2002). Cells were washed in PBS 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.

2.7. 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). 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 Q_{10}, 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).

2.8. Analysis of MMP

Changes in MMP were estimated by using the fluorescent cationic dye rhodamine 123 (Rh123), which preferentially partitions into active mitochondria based on the highly negative MMP. Depolarization of MMP results in the loss of Rh123 from the mitochondria and a decrease in intracellular fluorescence (Satoh et al., 1997). After cell treatment, culture medium in 24-well plates was removed and supplemented with 10 μM Rh123. Rh123 was incubated with cells for 30 min at 37 °C and then cells were washed with PBS to rinse the unconjuncted dye. The fluorescence was read in a fluorescence plat reader (Genios, TECAN) with excitation length 485 nm and emission length 530 nm.

2.9. Intracellular Ca^{2+} concentration assay

Intracellular Ca^{2+} concentration was measured by means of the fluorescent Ca^{2+} chelator Fura-2 AM, which permeated into cells where it was cut into Fura-2, resorting within cells. Fura-2 combined with intracellular Ca^{2+} to form a fluorescent compound, whose fluorescent intensity was determined at excitation wavelength 340 nm and emission wavelength 510 nm in a spectrophotometer (HITACHI, F-4500). After treatment, cells were harvested and rinsed with Krebs–Ringer buffer (137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES and 25 mM D-glucose, pH adjusted to 7.4). The harvested cells were suspended in Krebs-Ringer buffer and incubated with 5.0 μM Fura-2 AM for 60 min at 37 °C. During the session of incubation with Fura-2 AM, cell cultures were mildly shook at intervals of 10 min aimed to facilitate the combination of Fura-2 and Ca^{2+} to form the fluorescent compound. Then, cells were washed twice and resuspended in Krebs-Ringer buffer for fluorescent measurement.

2.10. Measurement of intracellular ROS

Formation of intracellular ROS was fluorometrically detected using the non-fluorescent probe DCFH-DA, which diffused into cells where it was oxidized in presence of ROS into the fluorescent compound 2′,7′-dichlorofluorescein (DCFH). Then, DCFH reacted with ROS to form the fluorescent product DCF. DCFH-DA was diluted in fresh DMEM/F12 at a final concentration of 10 μM and incubated with neuronal cells for 20 min at 37 °C. Later, cells were gently scraped. Fluorescent intensity was analyzed by a FACSCanto flow cytometer system (BD Biosciences).

2.11. The opening of MPT pore assay

The opening of MPT pore was determined by using commercially available kit (GENMED). All procedures completely complied with the manufacture's instructions. MPT pore opening was measured directly using a combination of calcein AM and $CoCl_2$. Calcein AM, which fluoresces upon binding with Ca^{2+}, was used to detect transient MPT pore opening in the high conductance mode of intact cells (Sharov et al., 2007). This was achieved by monitoring changes in mitochondrial Ca^{2+} levels in the presence of $CoCl_2$, which quenched the cytosolic Ca^{2+} signal produced by calcein. The fluorescence of calcein AM was monitored at the emission wavelength of 530 nm (FL-1), with the excitation wavelength being 488 nm. The extent of Ca^{2+}-induced MPT pore opening was estimated by noting the difference in fluorescence intensity between a certain phage.

2.12. Measurement of MAO-B activity

After treatment (in astrocyte cultures), samples were pre-incubated 30 min at room temperature with clorgyline (1 μM) as a specific MAO-A inhibitor. The fluorimetric assay started when 500 μl of a reaction mixture containing Amplex Red reagent (400 μM), horseradish peroxidase (2 U/ml) and benzylamine (2 mM) as a specific substrate for MAO-B were added. The assay was conducted in cuvette at room temperature for 45 min. At the end of the incubation time, specific fluorescence of resurofin, the oxidation product of Amplex Red reagent, was measured using a Bioteck Kontron cuvette fluorimeter (560 nm excitation and 590 nm emission).

2.13. Estimation of protein content

Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as standard.

2.14. Statistical analysis

Data are expressed as means ± S.D ($n = 4$) obtained from three independent experiments with $p < 0.05$ considered significant. Statistical significance was assayed by one-way analysis of variance (ANOVA) followed by Student's *t*-test.

3. Results

3.1. Neuroprotective effects of catalpol against MPTP-induced neurotoxicity in mesencephalic neuron-astrocyte cultures

The release of LDH and the cell viability were used as measurements of neurotoxicity on mesencephalic neurons. The results (Fig. 1) revealed that treatment with MPTP alone not only increased the release of LDH (51.2 ± 2.4%) but also had an apparent inhibitory effect on the cell viability (48.4 ± 3.0%). In contrast, pretreatment with catalpol significantly decreased the release of LDH (30.0 ± 1.6%), and these findings were further verified by MTT assay (87.5 ± 2.6%). Moreover, treatment with 0.5 mM catalpol alone did not affect the release of LDH and the cell viability (4.8 ± 1.6% and 102.1 ± 2.6%, respectively). Thus, it was possibly concluded that catalpol was effective for the protection of mesencephalic neurons.

3.2. Catalpol elevates mitochondrial complex I activity and MMP in mesencephalic neuron-astrocyte cultures induced by MPTP

It is well known the ETC complex I activity is selectively impaired in mitochondria by MPP^+ and lead to loss of the MMP (Schober, 2004). Apparently, catalpol attenuated MPTP-induced

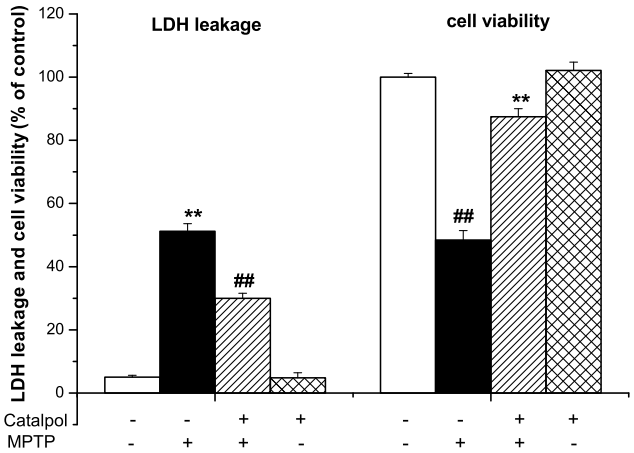

Fig. 1. Effects of catalpol on MPTP-induced cell damage in mesencephalic neuron-astrocyte cultures. Results are means ± S.D. from three independent experiments. $^{\#\#}p < 0.01$ in comparison with control, $^{**}p < 0.01$ compared with cells exposed to MPTP alone.

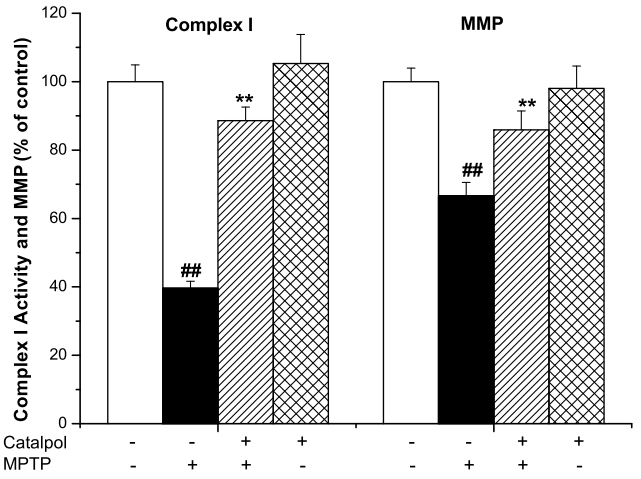

Fig. 2. Effects of catalpol on mitochondrial complex I activity and MMP in MPTP-treated mesencephalic neuron-astrocyte cultures. Results are means ± S.D. from three independent experiments. $^{\#\#}p < 0.01$ in comparison with control, $^{**}p < 0.01$ compared with cells exposed to MPTP alone.

mitochondrial complex I activity loss by 49% (Fig. 2). And it was unexpected that the cells treatment with catalpol alone obtained a notable enhancement on mitochondrial complex I activity by 5.3% compared with control.

As shown in Fig. 2 too, MMP produced a sharp fall that reached to 66.6 ± 3.9% of control after MPTP treatment, and then this potential maintained a steady-state level. However, catalpol prevented the significant collapse to 85.9 ± 5.5% that nearly resume to the normal level.

3.3. Catalpol blocks the accumulation of intracellular ROS and Ca^{2+} in mesencephalic neuron-astrocyte cultures induced by MPTP

There is evidence indicating ROS produced by MPP^+ in mitochondria could cause tissue damage (Duchen Michael, 2004). To examine the defensive effect of catalpol against oxidative attack, we detected the ROS levels in cells. As shown in Fig. 3, MPTP induced a marked increase in the ROS production (53.3%), whereas pre-treatment with catalpol showed a direct suppressive action on ROS formation that was reduced by 43.2%.

Like ROS, an increase in intracellular Ca^{2+} level is also a result of mitochondrial dysfunction. And that the overload of Ca^{2+} has been suggested to be the final common pathway of all type of cell death (Demaurex and Distelhorst, 2003). As compared with control group (Fig. 4), the concentration of intracellular Ca^{2+} was enhanced to 127.6 ± 3.7% in MPTP group. Nevertheless, the intracellular Ca^{2+} level was nicely attenuated by catalpol to 117.3 ± 4.1% of control. And there was no statistical significant change in intracellular ROS and Ca^{2+} level treated with catalpol alone.

3.4. Catalpol prevents the opening of MPT pore in mesencephalic neuron-astrocyte cultures induced by MPTP

The mitochondrial membrane permeability transition appears to be implicated in cell injury and death (Armstrong, 2006). Further, MPP^+ is found to induce the opening of membrane pores and cytochrome *c* release in brain and liver mitochondria (Cassarino et al., 1999).We found that MPTP treatment led to an evident opening of MPT pore approximately 116.2 ± 4.4% of control. Meanwhile, pre-treatment with catalpol slightly decreased the opening of MPT pore by 6.1% in comparison to MPTP groups (Fig. 5).

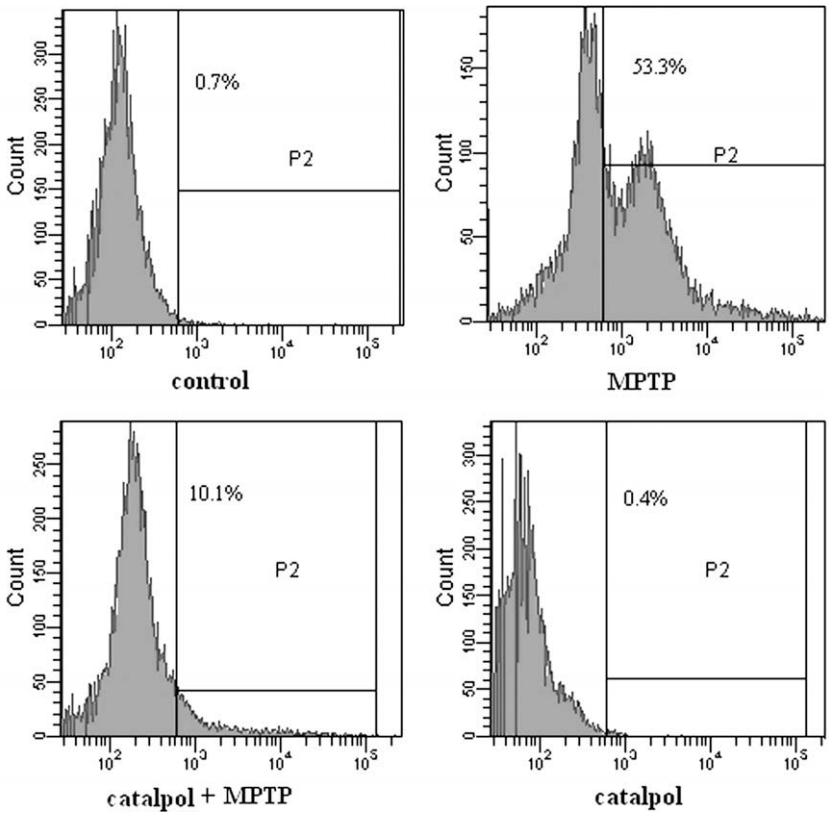

Fig. 3. Effects of catalpol on production of intracellular ROS in mesencephalic neuron-astrocyte cultures induced by MPTP. The level of intracellular ROS was determined by labeling with DCFH-DA and the fluorescent intensity was analyzed by flow cytometer.

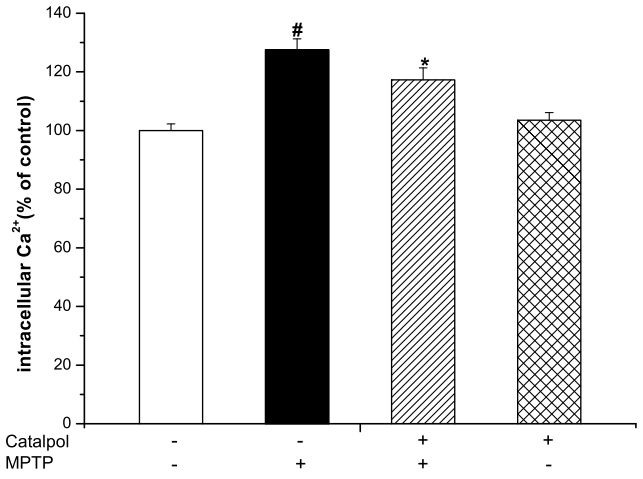

Fig. 4. Effects of catalpol on the elevation of intracellular Ca^{2+} level in mesencephalic neuron-astrocyte cultures induced by MPTP. Results are means ± S.D. from three independent experiments. $^{\#}p<0.05$ compared with control, $^{*}p < 0.05$ compared with cells exposed to MPTP alone.

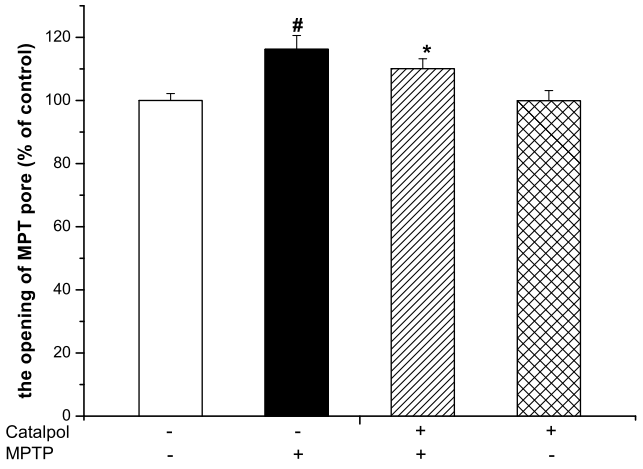

Fig. 5. Effects of catalpol on MPTP-induced the opening of MPT pore in mesencephalic neuron-astrocyte cultures. Results are means ± S.D. from three independent experiments. $^{\#}p < 0.05$ in comparison with control, $^{*}p < 0.05$ compared with cells exposed to MPTP alone.

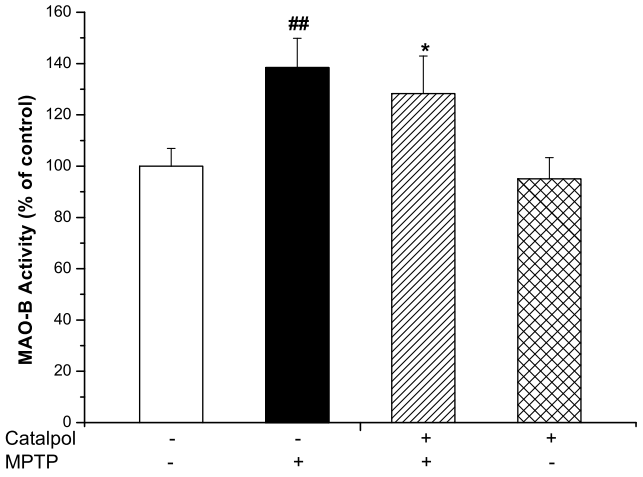

Fig. 6. Effects of catalpol on MAO-B activity in astrocytes cultures. Results are means ± S.D. from three independent experiments. $^{\#\#}p < 0.01$ in comparison with control, $^{*}p < 0.05$ compared with cells exposed to MPTP alone.

3.5. Catalpol inhibits MAO-B activity in astrocytes treated with MPTP

In order to determine if pre-treatment with catalpol could act by modifying MPTP pharmacokinetics, or by reducing the bioactivation of MPTP to MPP^+ or by reducing accumulation of MPP^+ in the DA neurons. We measured the activity of MAO-B in astrocyte cultures. The enzymatic assay showed a marked change in MAO-B activity between the MPTP group and catalpol pre-treatment group. As expected (Fig. 6), MAO-B activity was suppressed significantly by 10.2% compared with MPTP group, which may weaken the biotransformation of MPTP to MPP^+.

4. Discussion

PD is a debilitating neurological disorder that strikes approximately 2% of people over age 50 (Betarbet et al., 2000). Today, many new therapeutics and new strategies have emerged. However, drugs currently used for PD therapy only improve clinical symptoms, but hardly retard the progression of the disease. Moreover, side effects are often observed in the long-term treatment of PD. So, developing new drugs with better curative effect and fewer side effects for PD therapy is urgently needed. Recently, there is an increasing interest in focusing on natural products. Catalpol, separated from traditional Chinese herbal medicines has been shown to be neuroprotective in our laboratory (Li et al., 2005; Jiang et al., 2008). The mechanism of its action, however, needs further elucidation.

MPTP induces parkinsonism in many species and could be deprotonates to its toxic metabolite MPP^+ by MAO-B within astrocytes mostly (Squires, 1997). Once in the nervous system, the mechanism of action of MPP^+ is complex and incorporated into neurons leading to an inhibition of mitochondrial respiratory chain and mitochondrial dysfunction subsequently. Although it is still unclear whether mitochondrial dysfunction in DA neurons is the primary initiating event associated with neurodegeneration in PD, accumulated evidence have indicated its involvement at least in the propagation of cellular injury that leads to neuropathology in PD (Przedborski et al., 2004). Thereby, it is reasonable to consider that modulating mitochondrial dysfunction by drugs is an effective therapeutic target to protect neurons in neurodegenerative diseases.

In this paper, we noted that catalpol could protect against the MPTP-induced indirect injury to mesencephalic neurons by the release of LDH and the cell viability. In the mesencephalic neuron-astrocyte cultures, the addition of catalpol dramatically attenuated neurotoxicity induced by MPP^+ which converted by astrocytes. It was reported that MPP^+ directly acts as a mitochondrial chain inhibitor of complex I (Boada et al., 2000). And the conversion of the dye MTT to formazan crystals in cells has been shown to be related to mitochondrial redox state (Shearman et al., 1995) and respiratory chain activity (Musser and Oseroff, 1994). Therefore, it is quite possible that the loss of viability of mesencephalic neurons was subsequent to a serious mitochondrial dysfunction, and the neuroprotective action of catalpol may be via its protective effect on mitochondria. This speculation was based on the following evidence.

Inside the DA neurons, MPP^+ concentrates within the mitochondria, where it selectively impairs mitochondrial respiration chain by inhibiting complex I and MMP (Tretter et al., 2004). The inhibition of complex I activity by MPTP metabolite, MPP^+, is thought to be principal in the molecular mechanism of MPTP-induced neurotoxicity (Moore et al., 2005). It was also reported that critical events in cell apoptosis are the disruption of the MMP (Jellinger, 2000). Subsequently, damaged mitochondrial due to MPP^+ appears to cause a pathologic cascade involving both oxidative stress and

apoptosis in turn contributed to deterioration of DA neurons (Cadenas and Davies, 2000). The data of present study showed that the reduced complex I activity and the fall of MMP were significantly elevated by catalpol (which was consistent with the results from Tian et al.), indicating that catalpol might play a role in mitochondrial respiration chain regulation that may be also one of the main neuroprotective mechanisms of catalpol.

Much evidence had proved MPTP treatment resulting in ATP deficit, increased production of ROS and calcium imbalance from the respiratory chain, which initiates a deleterious cascade of events that result in cell death (Schober, 2004). In our laboratory, a lot of studies undertaken on cells and animals have shown that catalpol is capable of scavenging ROS and ameliorating antioxidant abilities through enhancing the antioxidant enzyme activities that protect them against oxidative damage (Li et al.,2004, 2008). In line with previous studies, catalpol was found to be scavenging ROS against oxidative attack in this experiment. In addition, we observed that the basal Ca^{2+} increased in the mesencephalic neuron-astrocyte cultures treated by MPTP alone. Calcium is an important second messenger and plays a crucial role in the modulation of apoptosis (Lee et al., 2006). And there is growing evidence for a possible link between an increase in Ca^{2+} and an enhanced generation of ROS (Mattson et al., 1995). Apparently, our data have validated this hypothesis. Similar to the inhibitory effect on ROS, catalpol also exerted a restraining effect on the overload of Ca^{2+}. Undoubtedly, it is a satisfying and exciting result. Because besides negatives above, there are other two deleterious effects of excessive Ca^{2+} accumulation at least, which it can lead to the opening of a high conductance pathway referred to the MPT pore and the deposition of Ca^{2+}–phosphate precipitates (Krieger and Duchen, 2002).

It is increasingly apparent that mitochondria lie at the centre of the process of cell death regulation and the opening of MPT pore contributes to mitochondrial dysfunction, resulting in osmotic swelling of mitochondrial matrix, dissipation of the membrane potential, cessation of the ATP synthesis, the release of cytochrome *c* and other apoptogenic factors (Crompton, 1999; Iverson and Orrenius, 2004). Thus, the opening of MPT pore is suggested to play a pivotal role in the degeneration of DA neurons. On this basis of concentration, neuroprotective effect of catalpol on the opening of MPT pore should be examined. Interestingly, catalpol exhibited a mild but adequate inhibitory effect on the opening of MPT pore which was promoted by MPTP. This outcome not only revealed the protection by catalpol on MPT pore involved mitochondrial dysfunction, but also implied the underlying relation between the excessive release of calcium from mitochondrial membrane and the opening of MPT pore. What's more, it is likely that the increased opening of MPT pore is one of the main causes for decreased MMP in this model.

It is well known that astrocytes play a neuroprotective role of structural and trophic support to the central nervous system (CNS) and owned an antioxidant defense mechanism because they contained superoxide dismutase, glutathione peroxidase, glutathione and vitamins C and E (Kirchhoff et al., 2001). On the other hand, their pivotal effect on the CNS is not negligible at all when MPTP administration because of the concentrated MAO-B in them (Shih and Chen, 1999). And mitochondrial MAO-B is considered to participate in the progress of nigrostriatal cell death (Ben-Shlomo and Bhatia, 2004). In this respect, the changes of MAO-B activity by catalpol were evaluated in astrocyte-enriched cultures. Consequently, catalpol effectively attenuated the MAO-B activity so that could rescue the DA neurons from more MPP^+-triggered neurotoxicity.

In summary, results from the two individual culture systems indicated that catalpol could protect mesencephalic neurons via attenuating mitochondrial dysfunction induced by MPTP in primary mesencephalic neuron-astrocyte cultures and suppressing MAO-B activity in astrocytes to reduce the neurotoxicity of MPP^+ conversed from MPTP. Further, our previous researches on PD models in vivo and vitro respectively confirmed that administration of catalpol significantly improved the abnormal behavior induced by rotenone (Mao et al., 2007) and protect DA neurons against MPP^+-induced oxidative stress (Tian et al., 2007). Together, it appears that catalpol is a potent neuroprotectant worthy for further research and development as a candidate drug of anti-PD.

Acknowledgements

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