Myricetin attenuated MPP⁺-induced cytotoxicity by anti-oxidation and inhibition of MKK4 and JNK activation in MES23.5 cells

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ABSTRACT

Increasing evidence suggests that oxidative stress may be implicated in the degeneration of dopaminergic neurons in Parkinson’s disease (PD), and anti-oxidation have been shown to be effective to PD treatment. Myricetin has been reported to have the biological functions of anti-oxidation, anti-apoptosis, anti-inflammation and iron-chelation. The aim of the present study is to investigate the neuroprotective effect of myricetin on 1-methyl-4-phenylpyridinium (MPP⁺)-treated MES23.5 cells and the underlying mechanisms. The results showed that myricetin treatment significantly attenuated MPP⁺-induced cell loss and nuclear condensation. Further experiments demonstrated that myricetin could suppress the production of intracellular reactive oxygen species (ROS), restore the mitochondrial transmembrane potential (ΔΨm), increase Bcl-2/Bax ratio and decrease caspase-3 activation that induced by MPP⁺. Furthermore, we also showed myricetin decreased the phosphorylation of mitogen-activated protein kinase (MAPK) kinase 4 (MKK4) and c-Jun N-terminal kinase (JNK) caused by MPP⁺. These results suggest that myricetin protected the MPP⁺-treated MES23.5 cells by anti-oxidation and inhibition of MKK4 and JNK activation.

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1. Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disorder characterized by selectively loss of dopaminergic neurons in the substantia nigra (SN), giving rise to dopamine (DA) depletion in the striatum. Although the precise pathogenic mechanism leading to neurodegeneration in PD is not known, a number of factors have been implicated in the pathogenesis of DA neuron loss. The generation of reactive oxygen species (ROS), mitochondrial dysfunction (Anantharam et al., 2007; Gardoni and Di Luca, 2006; Linazasoro, 2002; Mandel et al., 2000; McNaught et al., 2002), excitotoxicity (Gardoni and Di Luca, 2006; Moldzio et al., 2006) and inflammation (Koprich et al., 2008; Thomas et al., 2008) are all considered important mediators of neuronal death in PD. It is more likely that multiple factors converge to give rise to PD than any single reason.

The approaches to prevent dopamine neuron degeneration reflect the current notion on the pathogenesis of PD. Drugs directed against a single target will be ineffective and a single drug with multiple pharmacological properties maybe more suitable (Grunblatt et al., 2004; Van der Schyf et al., 2006; Youdim and Buccafusco, 2005), in which flavonoids are considered to be the multifunctional candidate because of their well-reported biological properties. Myricetin is one of such flavonoids. It has many biological functions, including anti-oxidation (Kang et al., 2010), anti-apoptosis (Shimmyo et al., 2008), free radical scavenging and anti-inflammation (Lee and Choi, 2008; Hladovec, 1977). Recently, several studies demonstrated the neuroprotective effects of myricetin. It has been shown that myricetin protect against rotenone-induced cytotoxicity in SHSY5Y cells (Molina-Jimenez et al., 2003). Myricetin also inhibits the formation and extension of amyloid-β protein for the prevention of Alzheimer’s disease (Ono et al., 2003). Myricetin also inhibits 6-hydroxydopamine-induced neurotoxicity in rats (Ma et al., 2007). However, the underlying mechanisms of myricetin-mediated neuroprotection are uncertain.

Abbreviations: MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; MPP⁺, 1-methyl-4-phenylpyridinium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; JNK, c-Jun N-terminal kinase; DA, dopamine; DMEM/F12, Eagle’s medium Nutrient Mixture-F12; ΔΨm, mitochondrial transmembrane potential; MKK4, mitogen-activated protein kinase kinase 4; PD, Parkinson’s disease; ROS, reactive oxygen species; RT-PCR, Reverse transcription-polymerase chain reaction; SN, substantia nigra.

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Activation of the mitogen-activated protein kinase (MAPK) kinase 4 (MKK4) and c-Jun N-terminal kinase (JNK) pathway has been shown to play a critical role in MPTP or 1-methyl-4-phenylpyridinium (MPP⁺) induced PD models (Xia et al., 2001). In the present study, we aim to elucidate whether myricetin could protect MPP⁺-induced neurotoxicity and the underlying mechanisms in MES23.5 cells.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals including myricetin were purchased from Sigma Chemical Co. The purity of myricetin used is 95%. Dulbecco’s modified Eagle’s medium Nutrient Mixture-F12 (DMEM/F12) was from Invitrogen. MES23.5 cells were cultured in DMEM/F12 containing Sato’s components growth medium supplemented with 5% fetal bovine serum, 100 U/mL of penicillin and 100 mg/mL of streptomycin at 37 °C, in a humid 5% CO₂, 95% air environment. For experiments, cells were seeded at a density of 1 × 10⁵/cm² in 24-well plates and treated as described above. After washing twice with cold PBS, cells were re-suspended in CitoLyse™ solution at a concentration of 1 × 10⁵ cells/0.5 mL and incubated for 20 min. The cells were then washed with Perm/Washing buffer twice and incubated in Perm/Washing buffer with antibody (1:5) for 30 min. After washing once with Perm/Washing buffer, the cells were re-suspended with 0.5 mL Perm/Washing buffer and analyzed by flow cytometry. The extent of apoptosis was determined by counting the number of active caspase-3 immunoreactive cells as a percentage of total MES23.5 cells using CellQuest Software.

2.2. Cell culture

MES23.5 cells were cultured in DMEM/F12 containing Sato’s components (3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were divided into vehicle group, MPP⁺ treatment group, MPP⁺ and myricetin co-treatment group. After incubation with the chemicals for 24 h respectively, the cells were incubated in MTT (5 mg/mL) for 4 h and cell viability was measured at 570 nm by colorimetric assay (Rayto RT-2100C, Shenzhen, China).

2.3. Cell viability assay

MES23.5 cells were seeded in a 96-well plate at a density of 2 × 10⁴ cells per well. Cell viability was measured by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were divided into vehicle group, MPP⁺ treatment group, MPP⁺ and myricetin co-treatment group. After incubation with the chemicals for 24 h respectively, the cells were incubated in MTT (5 mg/mL) for 4 h and cell viability was measured at 570 nm by colorimetric assay (Rayto RT-2100C, Shenzhen, China).

2.4. Hoechst 33258 staining

Nuclear morphology was detected using the method previously described in our lab (Xu et al., 2008; Zhang et al., 2005). MES23.5 cells were seeded on sterile cover glasses in 24-well plates and treated with vehicle, MPP⁺, MPP⁺ and myricetin for 24 h respectively. The cells were fixed in 4% paraformaldehyde for 30 min, washed in phosphate-buffered saline, and stained with Hoechst 33258 dye for 30 min at room temperature. After washing 3 times to remove the excessive dye, the cells were examined and photographed under a fluorescence microscope (Olympus, Japan) with an excitation wavelength of 330–380 nm. Apoptotic cells were defined on the basis of nuclear morphological changes, such as chromatin condensation and fragmentation. The total number of condensed cells was counted manually by researchers blinded to the treatment schedule using unbiased stereology. For each well, we delineated a 400 μm² field. Average sum of condensed and normal nuclei was calculated per well. The data were presented as the percentage of condensed nuclear number to the total number.

2.5. Reactive oxygen species (ROS) assay

Intracellular ROS in MES23.5 cells were measured by carboxy-H₂DCFDA as described before (Sanelli et al., 2007; Zhang et al., 2009; Zhu and Liu, 2004). The fluorescence coming from carboxy-H₂DCFDA reflects the intracellular ROS generation. The cells treated as described above were incubated in HEPES-buffered saline (HBS) containing carboxy-H₂DCFDA in a final concentration of 5 μmol/L for 30 min at 37 °C and followed by washing twice with HBS. Fluorescence intensity was recorded at 488 nm excitation and 525 nm emission wavelengths. Results were demonstrated as FL1-H (Fluorescence 1-Histogram), setting of the gated region M1 and M2 as a marker to observe the changing levels of fluorescence intensity by CellQuest Software.

2.6. Detection of mitochondrial transmembrane potential (ΔΨm)

The changes of mitochondrial membrane potential were measured by rhodamine123 using flow cytometry (Becton Dickinson, USA) as described before (Sanelli et al., 2007; Zhang et al., 2009; Zhu and Liu, 2004). The uptake of rhodamine123 into mitochondria is an indicator of ΔΨm. After 24 h treatment, the cells were incubated with rhodamine123 in a final concentration of 5 μmol/L for 30 min at 37 °C. The fluorescent intensity was recorded at 488 nm excitation and 525 nm emission wavelengths. Results were demonstrated as described in ROS assay.

2.7. Measurement of caspase-3 activation

The active caspase-3 was measured by PE-conjugated monoclonal active caspase-3 antibody apoptosis kit. Briefly, cells were seeded on 6-well plates and treated as described above. After washing twice with cold PBS, cells were re-suspended in CitoLyse™ solution at a concentration of 1 × 10⁵ cells/0.5 mL and incubated for 20 min. The cells were then washed with Perm/Washing buffer twice and incubated in Perm/Washing buffer with antibody (1:5) for 30 min. After washing once with Perm/Washing buffer, the cells were re-suspended with 0.5 mL Perm/Washing buffer and analyzed by flow cytometry. The extent of apoptosis was determined by counting the number of active caspase-3 immunoreactive cells as a percentage of total MES23.5 cells using CellQuest Software.

2.8. Western blot analysis

MES23.5 cells were incubated with vehicle, MPP⁺, myricetin, MPP⁺ and myricetin for 5 min respectively, then, cells were lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL aprotinin. Cell lysates were separated by 8% sodium dodecyl sulfate polyacrylamide gel and then transferred to polyvinylidene difluoride membrane. Blots were probed with anti-pMKK4 and anti-pJNK monoclonal antibody (Cell Signaling, 1:1000). Blots were also probed with anti-β-actin monoclonal antibody (Sigma, 1:10,000) as a loading control. Results were analyzed through scanning densitometry by Tanon Image System.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

MES23.5 cells were incubated with vehicle, MPP⁺, myricetin, MPP⁺ and myricetin for 24 h respectively, Total RNA was isolated by Trizol Reagent (Invitrogen). 5 μg of total RNA was reversed transcribed in a 20 μl reaction using the AMV reverse transcription system (Promega Corporation, Madison, WI, USA). We amplified Bcl-2 cDNA fragment (amplified products were 409 bp length) with the primers (forward: 5‘- GTC CCG CCT CTT CAC CTT -3‘; reverse: 5‘- CCC ACT GTG AGC CCC TTC T-3‘), Bax cDNA fragment (amplified products were 307 bp length) with the primers (forward: 5‘- GCC GAA TTT GAC ACG AAC AAA -3‘; reverse: 5‘- CGC AAG TAG CAG AGC AGG AGA -3‘) and GAPDH cDNA fragment (amplified products were 236 bp length) with the primers (forward: 5‘- TCC ACC ACC ATG AAG AAC GC -3‘; reverse: 5‘- GCC ATG TGC GGT CAT GA -3‘). The expression of house-keeping gene, GAPDH mRNA, was used as an internal standard. PCRs were run for 36 cycles in an Eppendorf Mastercycler. Denaturing, annealing, and extension reactions were performed at 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 45 s. The PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide, and detected by UV irradiation. The levels of Bcl-2 and Bax mRNA were expressed as their respective ratios to GAPDH mRNA.

2.10. Statistical analysis

Data were expressed as mean ± S.E.M. and analyzed using the SPSS11.5 software. One-way analysis of variance (ANOVA) followed by Tukey test was used to compare the differences between means. A probability value of less than 0.05 was considered to be statistically significant.
3. Results

3.1. Myricetin attenuated MPP⁺-induced cytotoxicity in MES23.5 cells

The cell viability was unchanged when treated with myricetin up to 50 μmol/L. However, a significant reduction of cell viability was observed when cells were treated with above 50 μmol/L myricetin (data not shown). Therefore, a concentration of lower than 50 μmol/L of myricetin was chosen to do the experiment. As shown in Fig. 1, the cell viability reduced significantly by 64% when treated with 200 μmol/L MPP⁺. Co-treated with 10⁻⁹ mol/L and 10⁻⁸ mol/L myricetin could significantly increase the cell viability compared to MPP⁺ treatment.

3.2. Myricetin attenuated MPP⁺-induced cell apoptosis indicated by Hoechst 33258

Morphological changes of the cells were further observed by Hoechst 33258 staining. As shown in Fig. 2, 200 μmol/L MPP⁺ treatment caused nuclear condensation in MES23.5 cells, 10⁻⁹ mol/L myricetin treatment significantly attenuated this effect.

3.3. Myricetin prevented the ΔΨm reduction in the MPP⁺-treated MES23.5 cells

Mitochondrial membrane potential is the marker of mitochondria function, which is involved in a variety of key events in oxidative stress and apoptosis. When treated with MPP⁺, MES23.5...
cells showed a markedly decrease of $\Delta \Psi_m$, this effect was partially reversed by myricetin (Fig. 3). This suggested that myricetin protect against MPP$^+$-induced cytotoxicity by restoring the mitochondria function.

3.4. Myricetin inhibited ROS production in the MPP$^+$-treated MES23.5 cells

ROS played an important role in cell apoptosis, the changes of $\Delta \Psi_m$ were considered to be involved in ROS production. We further measured ROS production using fluorescent dye H$_2$DCFDA. As shown in Fig. 4, 200 $\mu$mol/L MPP$^+$ treatment increased the production of ROS compared with that of control. Myricetin could markedly inhibit ROS production in the MPP$^+$-treated MES23.5 cells.

3.5. Myricetin inhibited caspase-3 activity in the MPP$^+$-treated MES23.5 cells

Caspase-3 is a key protein in the process of apoptosis. We further observed the caspase-3 activity in different treatment groups. Consistent with the above observation, increased caspase-3 activity was observed after treated with MPP$^+$. This effect was inhibited by treated with $10^{-9}$ mol/L myricetin (Fig. 5).

3.6. Myricetin inhibited the phosphorylation of MKK4 and JNK in the MPP$^+$-treated MES23.5 cells

The mechanisms of MPP$^+$-induced cell damage were also tested by measuring the MKK4 and JNK activity using anti-pMKK4 and anti-pJNK monoclonal antibody. As shown in Fig. 6, 200 $\mu$mol/L MPP$^+$ treatment significantly increased the phosphorylated MKK4 and JNK protein expressions. These effects were partially blocked by $10^{-9}$ mol/L myricetin. Treated by $10^{-9}$ mol/L myricetin alone has no effect on the activity of MKK4 and JNK.

3.7. Myricetin increased the ratio of Bcl-2/Bax in the MPP$^+$-treated MES23.5 cells

The Bcl-2 family is associated with mitochondrial function during apoptosis. Bcl-2, as an anti-apoptotic member of the Bcl-2 family, can bind Bax to form Bcl-2/Bax heterodimers, thereby attenuating the pro-apoptotic effect of Bax (Oltvai et al., 1993). In the present study, MPP$^+$ resulted in a markedly down-regulation of $\Delta \Psi_m$. 

![Fig. 4. Measurement of ROS production by flow cytometry in MES23.5 cells. 200 $\mu$mol/L MPP$^+$ treatment increased the production of ROS compared with that of control, $10^{-9}$ mol/L myricetin treatment protected against MPP$^+$-induced increase of ROS production. (A) Representatives of the fluorometric assay on ROS production in different groups. (B) Statistical analysis. Data were presented as mean ± SEM of 5 independent experiments. Fluorescence values of the control were set to 100%. ($**P < 0.01$, compared with the control; *$P < 0.05$, compared with MPP$^+$ treatment group).](image1)

![Fig. 5. Measurement of caspase-3 activity by flow cytometry in MES23.5 cells. 200 $\mu$mol/L MPP$^+$ increased the activity of caspase-3, this effect was inhibited by treated with $10^{-9}$ mol/L myricetin. (A) Representatives of the fluorometric assay on the activity of caspase-3 in different groups. (B) Statistical analysis. Data were presented as mean ± SEM of 4 independent experiments. Fluorescence values of the control were set to 100%. ($**P < 0.01$, compared with the control; *$P < 0.05$, compared with MPP$^+$ treatment group).](image2)
Bcl-2 mRNA and up-regulation of Bax mRNA in MES23.5 cells. Co-treatment with 10^{-9} mol/L myricetin could inhibit the decrease of Bcl-2 and increase of Bax caused by MPP\textsuperscript{+}. Therefore, the Bcl-2/Bax ratio in the MPP\textsuperscript{+}-treated cells significantly increased by myricetin (Fig. 7).

4. Discussion

In the present study, we demonstrated that myricetin effectively protected MES23.5 cells from MPP\textsuperscript{+}-induced cell death. The MES23.5 cells were chosen because it exhibits several properties similar to the primary neurons originated in the SN (Crawford et al., 1992). Therefore, the results from this cell line will give direct evident correlation to the degenerated dopamine neurons in PD. MPP\textsuperscript{+} is a common neurotoxin used as a chemical for inducing PD models. In this study, MPP\textsuperscript{+} injured the mitochondria function and increased ROS generation. When treated with myricetin, a fall in \Delta \Psi m was blocked and increased ROS level was decreased. These may attribute to the anti-oxidation and free radical scavenging properties of myricetin (Hanasaki et al., 1994). It is well known that MPP\textsuperscript{+} could enter the cell through the dopamine reuptake system, and then inhibit complex 1 of the mitochondrial respiratory chain, and may induce oxidative stress (Cassarino et al., 1999; Desai et al., 1996). Oxidative stress plays an important role in PD (Andersen, 2004; Jenner, 2003; Kidd, 2000). The dopamine-rich areas of the brain are particularly vulnerable to oxidative stress, because metabolism of dopamine itself leads to the generation of ROS, including hydrogen peroxide and hydroxyl radicals (Lotharius and Brundin, 2002). Mitochondrion is not only the main target of ROS, but also the major site of ROS production as well as primary target of oxidative molecular damage. The damaged mitochondrial membrane is implicated as key events in the pathogenic cascades; it breaks the balance of Bcl/Bax system and leads to cells death. Our study also showed that myricetin could partially reverse the MPP\textsuperscript{+}-induced dysfunction of Bcl/Bax system and then inhibit the activation of Caspase-3. These observations also highlighted that myricetin could protect the cells by anti-oxidation and mitochondria protection.

We further observed that the phosphorylated MKK4 and JNK protein levels markedly decreased after myricetin treatment compared with the MPP\textsuperscript{+} treatment group. It has been reported that activation of the JNK pathway plays a critical role in many models of neuronal degeneration. Inhibition of MKK4 and JNK activation could protect dopamine neurons in the MPTP or MPP\textsuperscript{+} models of PD (Herdegen and Waetzig, 2001; Xia et al., 2001). Although the exact protective mechanisms of myricetin on
dopamine neuron degeneration are still unclear, we hypothesize that the inhibitory effect of myricetin on MKK4 and JNK activation may play an important role. It has been shown that myricetin may bind MKK4 directly by competing with ATP and then trigger chemopreventive effects against TNF-α-related disease by inhibiting the MKK4 and downstream JNK activation (Kim et al., 2009). It seems likely that the protective action of myricetin on the toxicity of MPP⁺ may partly be attributed to the myricetin-suppressed MKK4 activity.

The simplified depiction of effects of myricetin on MPP⁺-induced cytotoxicity was summarized by Fig. 8. The in vitro neuroprotective action of myricetin may be mediated by a reduction of oxidative stress and an inhibition of MKK4 activation. However, in an in vivo situation, where myricetin is injected orally, it can suffer biotransformation altering its structure. Further studies should be carried out to test the in vivo protective action and try to find the promising metabolite of myricetin to treat PD. These studies may lead to new development of promising drugs for prevention and treatment of PD.

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