Original paper

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Mitochonic acid 5 ameliorates the motor deficits in the MPTP-induced mouse Parkinson's disease model by AMPK-mediated autophagy

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Abstract

Parkinson's disease (PD) is a well-known neurodegenerative disorder characterized by the degeneration of dopaminergic neurons, and oxidative stress and neuroinflammation are also associated with the pathogenesis of PD. Mitochonic acid 5 (MA-5), an analogue of indole-3-acetic acid, exerts key protective roles in inhibiting apoptosis, oxidative stress and neuroinflammation in multiple diseases. However, whether MA-5 can be beneficial for PD remains unclear. Hence, the aim of this study was to investigate the neuroprotective role of MA-5 in PD. In the current study, MPTP-challenged mice were treated as the in vivo model, and the effect of MA-5 on the motor function, neuronal survival, oxidative stress, neuroinflammation and the underlying mechanisms involved with AMPK and autophagy were determined. We revealed that MA-5 obviously up-regulated the phosphorylation of AMPK and promoted the autophagy (indicated by the increased LC3II/LC3I, parkin, pink and decreased p62) in substantia nigra (SN), ameliorated the motor deficits, up-regulated the expression of TH, suppressed the inflammation (indicated by the decreased protein levels of interleukin (IL)-1β, IL-6, tumour necrosis factor α) in SN in MPTP-induced mice. However, these patterns were reversed after the treatment of Compound C, an inhibitor of AMPK; also, after the application of CSA, an inhibitor of autophagy, MA-5 cannot play against the neurotoxicity of MPTP in mice. These combined results suggest that MA-5 can protect against MPTP-induced neurotoxicity to ameliorate the impaired motor function, which may be modulated via activation of AMPK-induced autophagy.

Key words: *Parkinson's disease (PD), mitochonic acid 5 (MA-5), AMPK, autophagy.*

Introduction

Parkinson's disease (PD), a common neurodegenerative disease in aging people, affects more than 4 million population around the world [4]. In this aging disorder, the degeneration of dopaminergic neurons was observed in substantia nigra pars compacta (SNpc), and resultant depletion of dopamine was detected in the striatum, accompanied by both motor and non-motor symptoms [13,14]. Current drug therapies for PD provide only symptomatic treatment and do not prevent the progressive loss of dopaminergic neurons in PD patients and concomitant

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decline [1]. It has been suggested that excessive generation of reactive oxygen species (ROS), oxidative stress, neuroinflammation, and mitochondrial dysfunction may account for the loss of dopaminergic neurons and neuronal apoptosis [30,36,52]. In this setting, to find an agent that can reduce the oxidative stress and inhibit neuroinflammation may be beneficial for the treatment of PD.

Mitochonic acid 5 (MA-5), an analogue of indole-3-acetic acid with its key roles in reducing neuroinflammation and preserving microglial function *via* synthesizing the indispensable neurotransmitters [38], is originally isolated from the plant [44], which primarily benefited from mitochondrial function mitochondrial function *via* reducing mitochondrial oxidative stress and accelerating mitochondrial energy metabolism [29]. MA-5 has been tested for treatment in patients with mitochondrial disease, cardiac myocyte damage and renal tubular injury [56]. MA-5 increases cellular ATP and protects mitochondrial patients' fibroblasts from cell death [44]. MA-5 also upregulates cardiac and renal respiration in the mitochondrial disease model [34]. Moreover, accumulating evidence indicates that MA-5 can attenuate the neuroinflammation and the apoptosis *via* activating the mitophagy [22,26,46].

Yet, since multiple mechanisms come into exert in PD, we then searched for other targets involved in MA-5 that can ameliorate PD. One of targets is autophagy, a pathway related to the degradation of organelles and protein [32], which is associated with the pathology of PD [5]. Autophagic dysfunction has been identified in various PD animal models and samples obtained from PD patients [28]. Accumulated evidence reveals that autophagy exerts critical roles in neuroprotection [15,55], for instance, the autophagic pathway can protect the survival of dopaminergic neurons via removing the synuclein in SNpc in PD models [2]. AMP-activated protein kinase (AMPK)/mTOR signalling, playing an essential role in neuronal survival and cell death [50,53], is associated with the regulation of autophagy in PD [12]. It has been demonstrated that activation of AMPK ameliorates the phenotypes of PD in Drosophila genetic models [35]. In addition, the induction AMPK-mediated autophagy by a multiple of drugs, including resveratrol and metformin, has recently been demonstrated to accelerate the functional recovery after spinal cord injury (SCI) [54,55].

Given the key neuroprotective roles of MA-5 under diseased conditions, we were interested in the effect of MA-5 on PD, hence, hypothesizing that MA-5 may exert a neuroprotective role in PD by fostering neuroinflammation *via* activating AMPK-mediated autophagy. In the present study, we reported a neuroprotective role of MA-5 against MPTP-induced neurotoxicity via activating AMPK-mediated autophagy, proposing that MA-5 is a novel candidate for the therapeutic strategy of PD.

Material and methods

Animals and groups

The 4-week-old male C57BL/6 mice with the body weight of 18 g were purchased from Hunan SJA Laboratory Animal CO., LTD and maintained (4/cage) in an air-conditioned room (22 ±1°C) with a 12 h light/12 h dark cycle and water and food *ad libitum*. All experimental protocols performed on animals were approved by the Laboratory Animal Ethics Committee of the First Affiliated Hospital, University of South China (Permit No. 20201226003).

C57BL/6 mice received i.p. injections of MPTP (30 mg/kg) in a volume of 10 ml/kg of body weight once daily for 7 days [43] and were randomly divided into 4 groups (n = 10/group): 1) MPTP + phosphate buffered saline (PBS) group treated with PBS; 2) MPTP + MA-5 group treated with MA-5; 3) MPTP + MA-5 + Compund C group treated with MA-5 and Compound C; and 4) MPTP + MA-5 + CSA group treated with MA-5 and CSA. The mice in the CTRL group were treated daily with 0.1 ml saline.

Open field test

The open field experiment is an efficient assay for evaluating the overall expression of motor deficits in mouse models of PD [41]. In this experiment, the open field consisted of a plaza box (50 × 50 cm), and a fence (40 cm tall). Mice were placed individually in the middle of the box and allowed to adapt to the new environment for a few minutes. Then, their behaviour was recorded on video for approximately 5 min. The box was cleaned with 70% alcohol and dried between each experiment to remove odour trails. The movement of the each mouse within 5 min was observed, and the total distance of movement was calculated. After performing the behavioural test, the mice were sacrificed.

Tissue preparation

Tissue preparation was performed according to the previous publications [6,8,9,49]. For western blot analysis, mice were sacrificed after anaesthesia by isoflurane. Briefly, the SN tissues (n = 3/group) were dissected and washed with 0.9% of ice-cold saline, and then, dissolved in 100 µl RIPA buffer with 1% PMSF and homogenised. The supernatants were collected after centrifugation at 14,000 g and 4°C for 15 min, and stored at –80°C for further analysis.

Western blot analysis

Western blot analysis was performed according to the previous publications [7,10,11,21,27,51]. The tissue

lysates mixed with a sample loading buffer were heated at 95°C for 15 min. Protein samples were subjected to 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes. After being incubated in 5% bovine serum albumin (BSA) diluted in Tris-HCl saline buffer supplemented with 0.1% Tween-20 (TBST, pH 7.4) for 1 h to block non-specific protein binding sites, membranes were incubated overnight at 4°C with one of the following antibodies: rabbit anti-AMPK antibody (1 : 1,000; ab133448, Abcam), rabbit anti-LC3A/B antibody (1 : 1,000; ab128025, Abcam), rabbit anti-P62 antibody (1 : 1,000; ab91526, Abcam), mouse anti-parkin antibody (1 : 1,000; ab77924, Abcam), rabbit anti-PTEN-inducible kinase 1 (PINK1) antibody (1 : 1,000; ab216144, Abcam), rabbit anti-β-actin (1 : 2,000; ab8227, Abcam). Then the membrane was washed with 0.1% TBST 3 times for 5 min each at RT, horseradish peroxidase-conjugated goat anti-mouse (1 : 10,000; ab6789, Abcam) or goat anti-rabbit secondary antibodies (1 : 10,000; ab97051, Abcam) diluted in TBST were incubated at RT for 1.5 h. Next, membranes were washed in 0.1% TBST 3 times for 5 min each at RT. The immunoreactive bands were visualized by an enhanced chemiluminescence (ECL) kit (170-5061, Bio-Rad Laboratories). The signal intensities were quantified by ImageJ 5.0 software.

Statistics

All statistical analyses were performed using GraphPad Prism 6 software. Data were expressed as mean ±SD and one-way ANOVA was performed followed by a post-hoc Bonferroni test. $P < 0.05$ was considered statistically significant.

Results

MA-5 up-regulates the phosphorylation of AMPK and promotes the autophagy in SN of MPTP-treated mice

To determine the effect of MA-5 on the AMPK phosphorylation and the mitophagy in SN of MPTP-treated mice, western blot was carried out to detect the p-AMPK level and LC3-I, LC3-II, P62, parkin, and PINK levels.

We observed that, in comparison to the CTRL group, the phosphorylation level of AMPK was down-regulated in the MPTP-treated group, but after the treatment of MA-5, the phosphorylation level of AMPK was up-regulated (Fig. 1A, B).

We also observed that, in comparison to the CTRL group, the ratio of LC3-II to LC3-I was decreased in the MPTP-treated group, but after the treatment of MA-5, the ratio of LC3-II to LC3-I was increased (Fig. 1C, D). The P62 level was down-regulated in response to the treatment of MPTP, but up-regulated in response to the treatment of MA-5 (Fig. 1C, E). In comparison to the CTRL group, the levels of parkin and PINK were decreased in the MPTP-treated group, but after the treatment of MA-5, the levels of parkin and PINK were increased (Fig. 1C, F, G).

MA-5 ameliorates the impaired motor function in MPTP-treated mice *via* activating the AMPK-mediated autophagy

To determine the effect of MA-5 on the recovery of the motor function in MPTP-treated mice, an Open Field Test was carried out and the total distance and average speed were calculated.

We observed that, in comparison to the CTRL group, the total distance and average speed were decreased in the MPTP-treated group, but after the treatment of MA-5, the total distance and average speed were increased, whereas, after inhibiting the AMPK and autophagy, MA-5 did not increase the total distance and average speed (Fig. 2A, B).

MA-5 up-regulates the expression of TH in SN of MPTP-treated mice *via* activating the AMPK-mediated autophagy

To determine the effect of MA-5 on the TH expression in SN of MPTP-treated mice, western blot was carried out to detect the TH level.

We observed that, in comparison to the CTRL group, the level of TH was down-regulated in the MPTP-treated group, but after the treatment of MA-5, the level of TH was up-regulated, whereas, after inhibiting the AMPK and autophagy, MA-5 did not increase the TH level (Fig. 3A, B).

MA-5 suppresses the inflammation in SN of MPTP-treated mice *via* activating the AMPK-mediated autophagy

To determine the effect of MA-5 on the inflammation in SN of MPTP-treated mice, western blot was carried out to detect the interleukin (IL)-1β, IL-6 and tumour necrosis factor α (TNF-α) levels.

We observed that, in comparison to the CTRL group, the levels of IL-1β, IL-6 and TNF-α were up-regulated in the MPTP-treated group, but after the treatment of MA-5, the levels of IL-1β, IL-6 and TNF-α were down-regulated, whereas, after inhibiting the AMPK and autophagy, MA-5 did not decrease the levels of IL-1β, IL-6 and TNF-α (Fig. 4A-C).

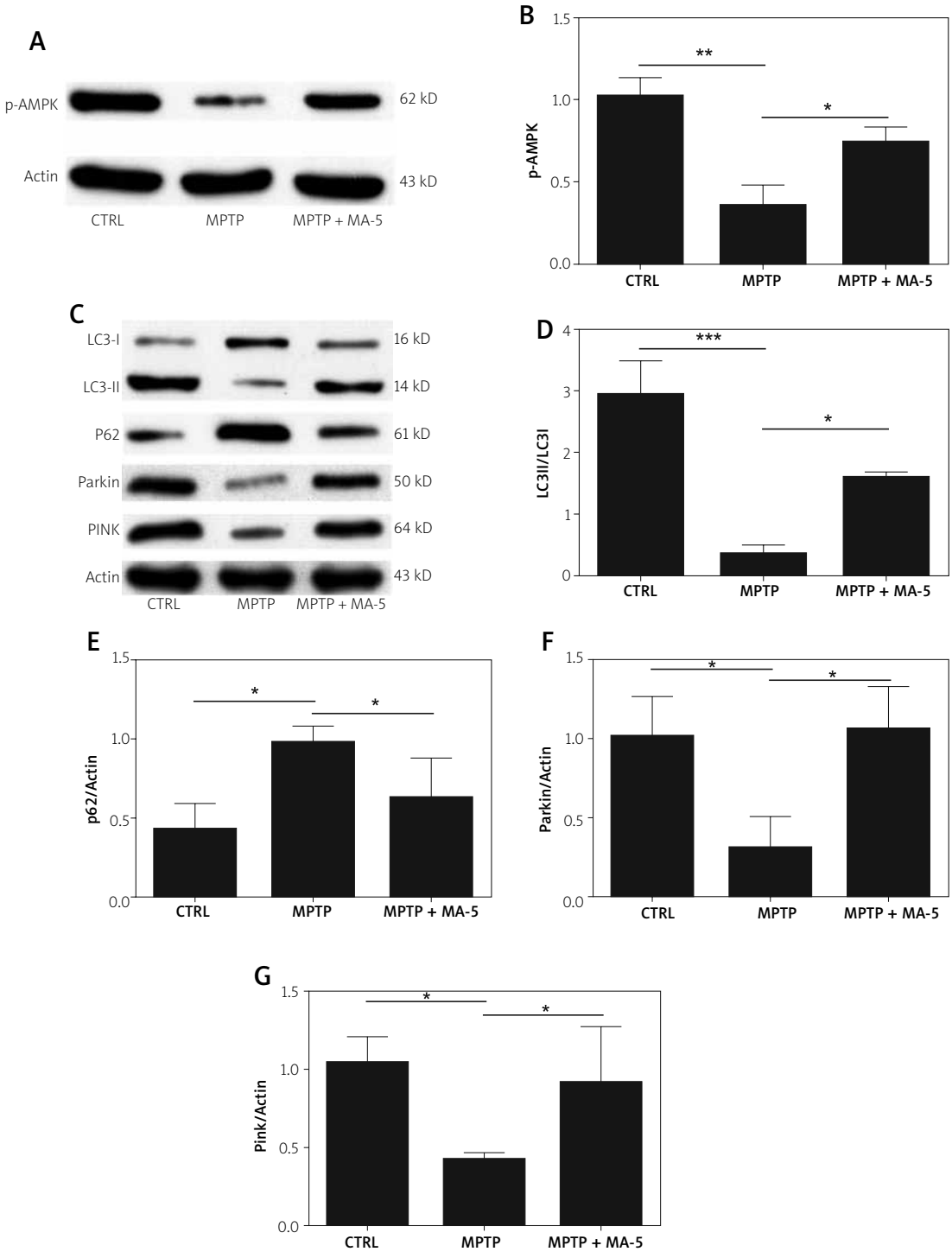

Fig. 1. Effect of MA-5 on the phosphorylation of AMPK and the autophagy in substantia nigra of MPTP-induced mice was determined by western blot. **A, B**) Phosphorylation of AMPK was up-regulated, and the autophagy was activated, indicated by (**C, D**) increased ratio of LC3II/LC3I, **C, E**) decreased *p62*, **C, F**) increased parkin, and (**C, G**) increased pink levels, in response to the treatment of MA-5. $^{**}p < 0.01$, $^{*}p < 0.05$, $n = 3$/subgroup.

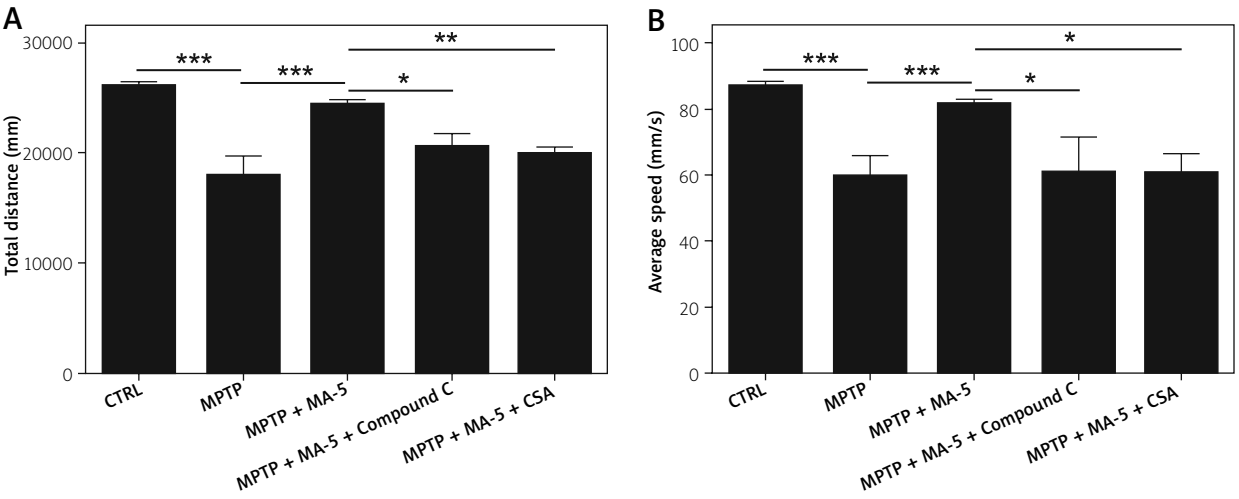

Fig. 2. Effect of MA-5 on the recovery of the motor function in MPTP-induced mice was determined by the Open Field Test. MA-5 promoted the motor impairments of MPTP-induced mice, indicated by (**A**) total distance and (**B**) average speed. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, $n = 6$/subgroup.

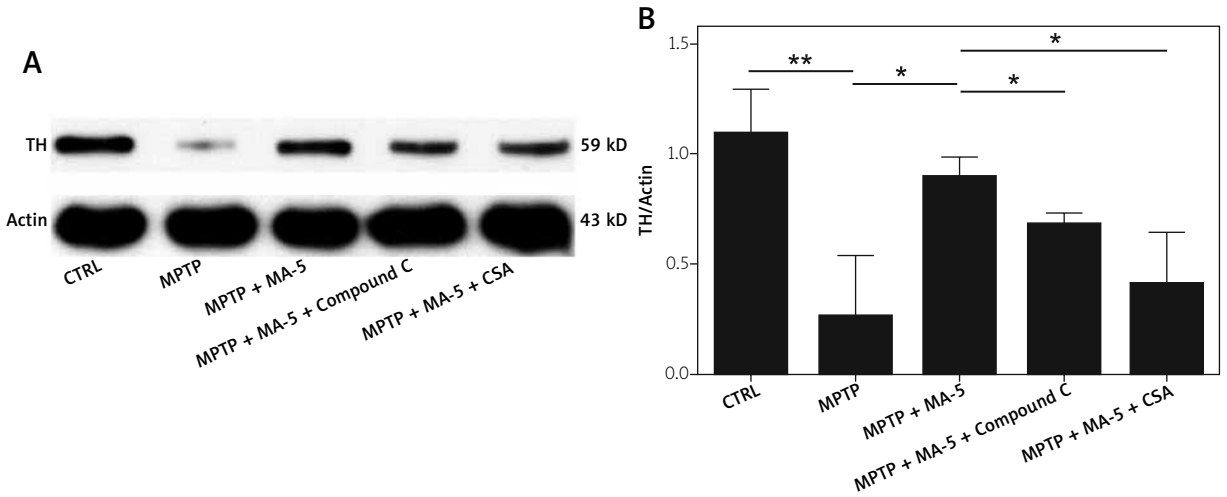

Fig. 3. Effect of MA-5 on the TH level of substantia nigra (SN) of MPTP-induced mice was determined by western blot. The TH level in SN was up-regulated in response to the treatment of MA-5. **$p < 0.01$, *$p < 0.05$, $n = 5$/subgroup.

Discussion

In the previous studies, we demonstrated that MA-5 can exert beneficial effects on mitochondrial homeostasis and increase microglial apoptosis by regulating mitophagy *via* Bnip3 through the MAPK-ERK-Yap signalling pathway [26] and promote the survival of microglial cells *via* Mitofusin 2-related mitophagy in response to lipopolysaccharide-induced inflammation [46]. In this study, we revealed that MA-5 can ameliorate the impaired motor function under MPTP-induced neurotoxicity *via* activating AMPK-mediated autophagy.

The dysfunctional autophagy has been well-known in relation to PD [31]. AMPK, acting as an energy sensor in response to stress conditions, including oxidative stress and nutrition deprivation, serves as an important modulator in the development of autophagy [25]. LC3 and P62 proteins are wide acknowledged to performed to monitor the autophagic flux [37]. The LC3-II/LC3-I, an indicator of autophagy status [42], is significantly up-regulated in PD [20]. P62, a proteolytic substrate in autophagy, is down-regulated with the increase of autophagy [40], and is down-regulated in PD [48]. To date,

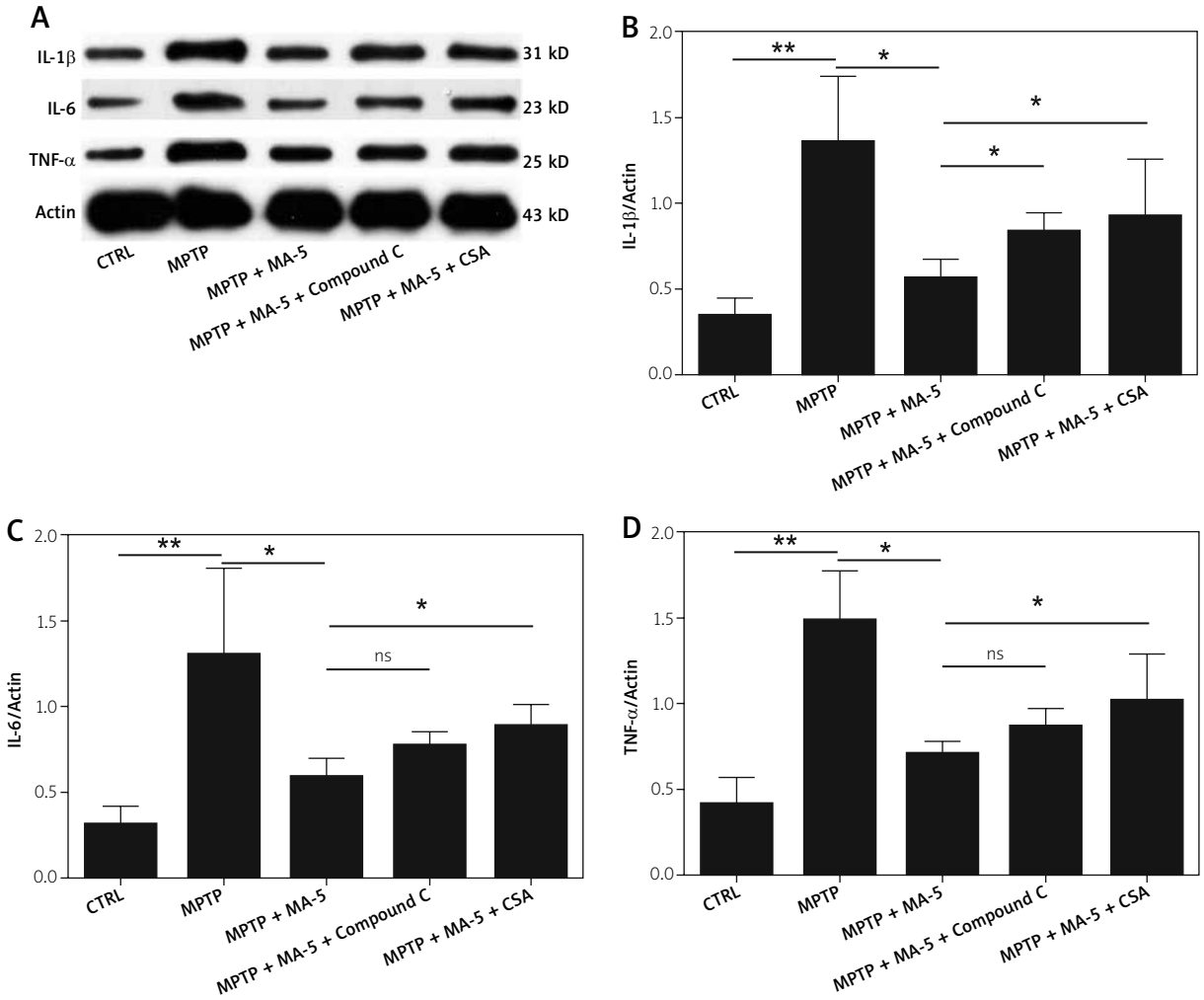

Fig. 4. Effect of MA-5 on the inflammation in substantia nigra of MPTP-treated mice was determined by western blot. The levels of proinflammatory cytokines including: **A**) IL-1β, **B**) IL-6, **C**) TNF-α were down-regulated in response to the treatment of MA-5. $^{**}p < 0.01$, $^{*}p < 0.05$, $n = 5$/subgroup.

the most well-known mitophagy pathway, has been mediated by PINK1 and Parkin, representing a crucial amplifying mechanism that renders mitophagy more efficient [18]. In the present study, we observed that MA-5 can promote the phosphorylation of AMPK and the autophagy in mice induced by MPTP.

As we all know, neurological function rehabilitation is beneficial for protecting against further worsening under the pathological condition of PD patients [45]. Assessment of the neurological function is widely carried out to determine the therapeutic effect of strategies. Multiple studies have reported that behavioural tests can be performed to evaluate the motor dysfunction in the MPTP-induced PD mouse model [39]. Locomotor dysfunction serves as a wide-acknowledged clinical symptom of PD [23]. In our previous study, we observed that dietary tryptophan can ameliorate the impaired motor function in PD [47], in the current study, we revealed that MA-5 can promote the recovery of the motor function in mice induced by MPTP *via* activating the AMPK-mediated autophagy.

The main origin of PD and its related impaired motor function is the degeneration of dopaminergic neurons [19]. The lack of TH, a specific marker of dopaminergic neurons, is thought to contribute to the progression of PD [24]. TH, a rate-limiting enzyme during biosynthesis of L-dihydroxyphenylalanine (L-DOPA), is closely associated with the motor function [24]. In the current study, we revealed that MA-5 can up-regulate the TH level in SN of mice induced by MPTP *via* activating the AMPK-mediated autophagy.

Concentrations of IL-1β and IL-6 in SNpc and blood are significantly higher in PD than age-matched subjects without any neurological disease [33]. Subse-

quently, it has been shown that the secretion of IL-1β, IL-6, and TNF-α is significantly enhanced in peripheral blood mononuclear cells of PD patients compared with age-matched controls [3]. Previous studies have also indicated that the survival of dopaminergic neurons could be protected *via* inhibiting the microglia-related neuroinflammatory responses [16,17]. In the present study, we observed that MA-5 can inhibit the expression levels of IL-1β, IL-6 and TNF-α in mice induced by MPTP *via* AMPK-mediated mitophagy *via* activating the AMPK-mediated autophagy.

In conclusion, MA-5 can exert a beneficial effect on PD, at least in part, via the AMPK-mediated autophagy, laying the foundation for providing invaluable therapeutic strategies for the treatment of PD.

Although the results seem promising, our study still exhibited some limitations. Further studies are no doubt needed to be performed to detect the survival rate of dopaminergic neurons using histochemical staining. All in all, MA-5 may be a novel candidate for the treatment of PD.

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Disclosure

The authors report no conflict of interest.

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