Asiatic Acid Protects Dopaminergic Neurons from Neuroinflammation by Suppressing Mitochondrial ROS Production

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Abstract
This study sought to evaluate the effects of Asiatic acid in LPS-induced BV2 microglia cells and 1-methyl-4-phenyl-pyridine (MPP⁺)-induced SH-SY5Y cells, to investigate the potential anti-inflammatory mechanisms of Asiatic acid in Parkinson’s disease (PD). SH-SY5Y cells were induced using MPP⁺ to establish as an in vitro model of PD, so that the effects of Asiatic acid on dopaminergic neurons could be examined. The NLRP3 inflammasome was activated in BV2 microglia cells to explore potential mechanisms for the neuroprotective effects of Asiatic acid. We showed that Asiatic acid reduced intracellular production of mitochondrial reactive oxygen species and altered the mitochondrial membrane potential to regulate mitochondrial dysfunction, and suppressed the NLRP3 inflammasome in microglia cells. We additionally found that treatment with Asiatic acid directly improved SH-SY5Y cell viability and mitochondrial dysfunction induced by MPP⁺. These data demonstrate that Asiatic acid both inhibits the activation of the NLRP3 inflammasome by downregulating mitochondrial reactive oxygen species directly to protect dopaminergic neurons from, and improves mitochondrial dysfunction in SH-SY5Y cells, which were established as a model of Parkinson’s disease. Our finding reveals that Asiatic acid protects dopaminergic neurons from neuroinflammation by suppressing NLRP3 inflammasome activation in microglia cells as well as protecting dopaminergic neurons directly. This suggests a promising clinical use of Asiatic acid for PD therapy.

Key Words: Parkinson’s disease, Neuroinflammation, Asiatic acid, NLRP3 inflammasome, Mitochondria

INTRODUCTION
Parkinson’s disease (PD) is the second most common neurodegenerative movement disorder after Alzheimer’s disease (AD), and is characterized by progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and accumulation of α-synuclein (α-syn) in Lewy bodies (Dauer and Przedborski, 2003; Moore et al., 2004). Despite the fact that the intricate etiology of PD has not been fully elucidated, a crucial relationship has been determined between PD and inflammation (Hirsch et al., 2005; Whitton, 2007). High levels of the proinflammatory cytokines, IL-1β and tumor necrosis factor α (TNF-α), have been detected in the postmortem brains of PD patients (Kalia and Lang, 2015; Ellis and Fell, 2017). Cytoplasmic multiproteins known as “inflammasomes” which contain nod-like receptor protein 3 (NLRP3), adaptor apoptosis-associated speck-like protein (ASC), and proinflammatory mediators caspase-1 and IL-1β, control the activity of IL-1β and IL-18 (Schroder and Tschopp, 2010). Recently, researchers have demonstrated that a fundamental component of characteristic PD pathology, fibrillar α-synuclein, may induce the production of IL-1β, which is involved in NLRP3 inflammasome activation (Codolo et al., 2013). Interestingly, the NLRP3 inflammasome is not only involved in regulating DA neuron survival, but also participates in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced loss of DA neurons (Yan et al., 2015), suggesting that the NLRP3 inflammasome has a relationship with PD. 1-methyl-4-phenyl-pyridine (MPP⁺) is a toxic component of MPTP that is converted by monoamine oxidase in glial cells (Chiba...
et al., 1964). MPP⁺ selectively enters the DA neurons located in the substantia nigra via the presynaptic dopamine transporter (DAT), causing oxidative stress, mitochondrial dysfunction and programmed cell death (Lambert and Bondy, 1989; Schapira, 1999). Therefore, MPP⁺ is widely used in in vitro and in vivo models of PD (Singer and Ramsay, 1990).

Asiatic acid (AA) is one of the main triterpene components of Centella asiatica (L.) Urban. Clinical examinations have demonstrated protective effects of AA in the liver, nervous system and skin (Shetty et al., 2006; Tang et al., 2012; Zhang et al., 2012; Lu et al., 2016; Wu et al., 2017). Researchers have shown that AA can increase glutamate-induced brain damage and worsen memory function in mice (Xu et al., 2012). AA derivatives have been shown to counteract glutamate-induced excitotoxicity in nerve cells (Lee et al., 2000), while asiaticoside derivatives can antagonize β-amyloid-induced nerve damage (Jew et al., 2010; Mook-Jung et al., 2015). Furthermore, AA shows neuroprotective effects against aluminum-induced neuronal toxicity in vivo and in vitro (Ahmad et al., 2018a, 2018b). Xiong has shown that rotenone or H₂O₂ can reduce the mitochondrial membrane potential (MMP) of SH-SY5Y DA cells. However, AA can prevent rotenone or H₂O₂-induced SH-SY5Y cell damage, restore MMP, and reduce VDAC expression (Xiong et al., 2009). Chao reported that AA had a protective effect in an MPTP-induced PD model in mice via various pathways including anti-oxidation, anti-inflammation and anti-apoptosis (Guo et al., 2015; Chao et al., 2016).

Despite the fact that AA has demonstrated critical anti-inflammatory effects in the treatment of diseases such as PD, its anti-inflammatory mechanisms have not yet been clarified. Considering that the NLRP3 inflammasome plays a pivotal role in the inflammatory mechanism of neurodegenerative diseases like PD, whether AA directly targets the NLRP3 inflammasome and inhibits activation to prevent PD warrants investigation. Taken together, we hypothesized that the protective mechanism of AA against PD might be through the inhibition of NLRP3 inflammasome activation, diminishing the inflammatory response to alleviate and improve PD symptoms.

**MATERIALS AND METHODS**

**Cell culture and treatment**

Human neuroblastoma SH-SY5Y cells were maintained in minimum Eagle’s medium (MEM; Gibco/Invitrogen, CA, USA) containing 1% nonessential amino acids (Gibco, CA, USA) and 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). LPS (10 μg/ml) was used as a positive control for BV2 microglia cell damage. BV2 microglia cells were treated with AA and viability was assessed using the MTT assay. Data are expressed as mean ± SD, analyzed by one-way ANOVA, *p<0.05 compared with the control group; **p<0.01 compared with the model group.

**Fig. 1.** Neuroprotective effects of AA on LPS-induced BV2 microglia cell damage. (A) Dose-dependent toxic effects of LPS on viability of BV2 microglia cells. (B) BV2 microglia cells were treated with AA and viability was assessed using the MTT assay. (C) BV2 microglia cells were pre-treated with AA for 24 h before being treated with LPS and viability was assessed using the MTT assay. Data are expressed as mean ± SD, analyzed by one-way ANOVA, *p<0.01 compared with the control group; **p<0.01 compared with the model group.

**Fig. 2.** AA inhibits NLRP3 inflammasome activation in microglia cells. (A-F) Immunoblot analysis of BV2 cells treated for 24 h with various doses of AA following stimulation with LPS (10 μg/ml) for 24 h, immunoblot analysis of NLRP3, the precursors of IL-1β (pro-IL-1β) and caspase-1 (pro-caspase-1), cleaved caspase-1 (c-caspase-1(20)), and IL-1β in lysates of those cells. Data are expressed as mean ± SD, analyzed by one-way ANOVA, *p<0.05 and **p<0.01 compared with the control group; *p<0.05 and **p<0.01 compared with the model group.
streptomycin (100 U/ml) in a humid atmosphere of 5% CO₂ and 95% air at 37°C. Murine BV2 microglia cells were cultured in DMEM/F12 (1:1; Gibco/Invitrogen) media supplemented with 10% FBS. Prior to experiments, SH-SY5Y cells and BV2 cells were seeded in 96-well plates at a density of 3×10⁴ viable cells per well (MTT assay). After 24 h, cells were treated with AA at a concentration of 0.1, 1.0, or 10 nM for 24 h. Then, SH-SY5Y cells were exposed to MPP⁺ at a concentration of 200, 300, 400 or 500 μM (Sigma Aldrich, USA) for 24 h. BV2 cells were stimulated by LPS (Sigma Aldrich, MO, USA) at a concentration of 0.1, 1.0, or 10 μg/mL for 24 h.

**MTT assay**

Briefly, 100 μL/well MTT (5 mg/ml, dissolved in PBS solution; Sigma Aldrich) was added to 96-well plates. After incubating for 4 h, 100 μL/well DMSO solution was added to each well and plates were placed on a shaker for 10 min. Absorbance was detected using a multi-plate reader at a wavelength of 570 nm (A₅₇₀).

**Western blot analysis**

Microglia cells were seeded in 6-well plates. First, adherent cells were washed twice with 1X PBS and collected by centrifugation at 600 g. The precipitate was lysed with 100 μL RIPA lysis buffer (containing 10 μL protease inhibitor cocktail tablets, each tablet dissolved in 1 mL RIPA solution, Roche, Mannheim, Germany). Lysates were centrifuged at 12,500 g for 15 min at 4°C to obtain supernatants of the whole cell lysates. Protein concentration was measured using a bicinechonic protein assay (Beyotime Biotechnology, Shanghai, China). Proteins were electrophoresed through a 10-15% SDS-polyacrylamide gel and transferred to a polyvinylidene-difluoride (PVDF) membrane (Bio-Rad, Billerica, MA, USA). Blots were probed with the following primary antibodies (Cell Signaling Technology, MA, USA): anti-NLRP3 (1:1000), anti-caspase-1 (1:500), anti-IL-1β, anti-α-actin (1:1000) and anti-β-actin (1:1000). The precipitate was lysed with 100 μL lysis buffer (Sigma Aldrich) was added to 96-well plates. After incubation for 4 h, 100 μL/well DMSO solution was added to each well and plates were placed on a shaker for 10 min. Absorbance was detected using a multi-plate reader at a wavelength of 570 nm (A₅₇₀).

**Detection of intracellular ROS formation**

2,7-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Invitrogen, CA, USA) was used to detect the production of intracellular ROS. Cells were seeded in a 6-well plate at a concentration of 2.0×10⁵/mL and stimulated by LPS or MPP⁺ with or without pre-incubation with AA for 24 h. After 24 h, cells were washed twice with PBS and the intracellular ROS levels were detected using a fluorescence microscope.

**Detection of mitochondrial ROS production**

MitoSOX red mitochondrial superoxide indicator (Molecular Probes, Invitrogen) was used to assess mitochondrial ROS (mtROS) production. Cells were cultured in a 6-well plate at a concentration of 2.0×10⁵/mL and stimulated by LPS or MPP⁺ with or without pre-incubation with AA for 24 h. After treatment, cells were treated with 5-μM MitoSOX red for 20 min at 37°C. Cells were then washed twice with PBS and the intracellular ROS levels were detected using a fluorescence microscope.

**Detection of IL-1β and TNF-α release**

BV2 microglia cells were seeded in 96-well plates at a density of 3×10⁴/mL viable cells per well, and then treated with different stimuli. The concentrations of IL-1β and TNF-α in the supernatants from BV2 microglia cells were pre-treated with various doses of AA for 24 h and then stimulated with LPS (10 μg/mL). After 24 h, the media was changed and cells were cultured for 24 h. Data are expressed as mean ± SD, analyzed by one-way ANOVA, *p<0.05 and **p<0.01 compared with the control group; *p<0.05 and **p<0.01 compared with the model group.
cell culture supernatant were measured using an ELISA kit (Thermo Fisher Scientific, CA, USA).

**Statistical analysis**

All data are expressed as the mean ± SD and were analyzed by one-way ANOVA or Student’s t test as appropriate, using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The p<0.05 was considered significant.

**RESULTS**

**Cell toxicity of AA in BV2 microglia cells**

Initial experiments were performed to determine whether AA or LPS was toxic to BV2 microglia cells. As shown in Fig. 1A, LPS induced dose-dependent cell cytotoxicity. In order to estimate the neuronal cytotoxicity of AA, BV2 microglia cells were treated with various concentrations of AA (0.1-100 nM; Fig. 1B) for 24 h. As a result, the concentration of 10 μg/mL of LPS was determined to induce BV2 microglia damage in subsequent experiments.

To investigate the protective effects of AA against LPS-induced loss of neuronal cell viability, BV2 microglia cells were pre-treated with AA (0.1-10 nM) for 24 h, followed by treatment with 10 μg/mL LPS for 24 h. LPS-induced cell damage was significantly ameliorated by 10 nM of AA pretreatment (p<0.01; Fig. 1C).

**AA suppresses the expression of NLRP3 to protect microglia**

We next assessed the mechanisms responsible for the protective effects of AA against LPS-induced microglial damage. LPS induced caspase-1 activation and increased IL-1β production in microglia. The expression of inflammasomes such as IL-1β, NLRP3, and caspase-1 were downregulated by AA in a concentration-dependent manner (Fig. 2A-2F). In particular, western blot and ELISA analyses revealed that the inhibition of IL-1β and TNF-α secretion in cell supernatants also occurred in a concentration-dependent manner (Fig. 3A, 3B). These results demonstrate that AA can suppress the activation of NLRP3 inflammation in BV2 microglia cells. To investigate the potential molecular mechanism, we used the DCFH-DA fluorescent probe to detect the production of cell reactive oxygen species (ROS) in BV2 microglia cells, which is considered an activator of the NLRP3 inflammasome. As shown...
in Fig. 4, LPS markedly increased ROS in BV2 microglia cells (Fig. 4G, p<0.01). However, when BV2 microglia cells were pre-treated with various concentrations of AA, ROS production showed a significant concentration-dependent decrease (Fig. 4G; p<0.05, p<0.01). These results suggest that LPS can activate the NLRP3 inflammasome by increasing the production of ROS in BV2 microglia cells, and AA is a potent inhibitor of NLRP3 inflammasome activation.

**AA protects microglia from mitochondrial dysfunction**

The induction of oxidative stress in neurons leads to mitochondrial dysfunction (Niizuma et al., 2010). Therefore, we assessed whether LPS changed mtROS and MPP. LPS increased the production of mtROS. However, BV2 microglia cells that were pretreated with various concentrations of AA had significantly decreased production of mtROS (Fig. 5G; p<0.05, p<0.01). Meanwhile, LPS increased the green-to-red fluorescence ratio of the mitochondria binding fluorochrome JC-1. Cells pretreated with AA had decreased green-to-red fluorescence ratios (Fig. 6F; p<0.05, p<0.01). This indicates that mitochondrial dysfunction in BV2 microglia cells caused by LPS injury can be blocked by AA.

**Neuroprotective effects of AA on MPP+-induced cell damage**

Microglial activation and neuronal damage are two notable mechanisms leading to PD (Damier et al., 1999; Rose et al., 2017; Sarkar et al., 2017). We demonstrated that AA can prevent BV2 microglia cells from LPS-induced inflammation by protecting against NLRP3 inflammasome activation from mtROS. Along these lines, we investigated whether AA had a protective effect on neuronal damage in PD. We chose the SH-SY5Y cell line as a model system for neuron cultures. SH-SY5Y cells were exposed to various concentrations of MPP+ (200-500 μM) for 24 h to assess toxicity. As shown in Fig. 7A, MPP+ was toxic to SH-SY5Y cells at concentrations over 300 μM (p<0.01). No toxicity was observed in SH-SY5Y cells following treatment with various concentrations of AA (0.1-10 nM) (Fig. 7B). Interestingly, following pretreatment with AA for 24 h, MPP+ did not cause any significant damage to SH-SY5Y cells (Fig. 7C). These data suggest that AA had protective effects on MPP+-induced damage to SH-SY5Y cells. Further, we sought to verify whether AA protected SH-SY5Y cells from inflammasomes such as IL-1β and TNF-α. As shown in Fig. 7D, AA protected SH-SY5Y cells from IL-1β and TNF-α (p<0.05).

**AA protected SH-SY5Y cells from mitochondrial dysfunction**

To determine whether AA had neuroprotective effects
against MPP+-induced mitochondrial dysfunction in PD, we analyzed mtROS and MMP using MitoSOX red and JC-1 fluorochrome. As shown in Fig. 8G, MPP+ significantly increased the production of mtROS in SH-SY5Y cells (p<0.01), and the groups that were pre-treated with AA had significantly decreased levels of mtROS (p<0.05, p<0.01). Accordingly, MPP+ alone resulted in significant loss of MMP, and pretreatment of cells with AA resulted in increased fluorescence compared with that in cells treated with MPP+ alone (Fig. 9G; p<0.05, p<0.01), indicating that AA protected SH-SY5Y cells from the loss of MMP caused by MPP+.

**DISCUSSION**

Oxidative stress has long been known to be involved in an assortment of neurodegenerative diseases (Al-Ayadhi, 2004; Xu et al., 2015). The present study was performed to determine whether AA had neuroprotective effects on both microglia and DA neurons in PD. We demonstrated that AA exerted partial neuroprotective effects in PD. AA pretreatment protected BV2 microglia cells from LPS-induced neuroinflammation damage, and protected DA neurons from MPP+-induced PD-like damage. We additionally found that AA appeared to protect BV2 microglia cells from LPS-induced neuroinflammation by regulating mitochondrial dysfunction, and protected SH-SY5Y cells from MPP+-induced mitochondrial dysfunction, indicating the potential of AA as a prospective therapy for PD.

In the present investigation, we found that increasing the concentration of LPS in BV2 microglia cells caused a dose-dependent reduction in cell viability. However, pretreatment with AA markedly attenuated the toxic effects 10 μg/mL LPS treatment for 24 h. Increasing evidence has demonstrated that the NLRP3 inflammasome is involved in the progression of PD (Fan et al., 2015; Qiao et al., 2016; Zhou et al., 2016). Two signals are required in NLRP3 inflammasome-mediated IL-1β production. One activates the NLRP3 inflammasome to mediate caspase-1 cleavage specifically, which results in the maturation of IL-1β. The other is a prerequisite for inflammasome activation, which induces nuclear transcription factor-κB (NF-κB) to increase the expression of NLRP3 and proIL-1β (Latz, 2010; Liu et al., 2013). In order to assess the effect of AA on the NLRP3 inflammasome, the NLRP3 inflammasome was activated by LPS in BV2 microglia cells. We determined that treatment with appropriate doses of AA significantly reduced release of IL-1β in BV2 microglia cells. In addition, AA inhibited caspase-1 cleavage and reduced the expression of NLRP3 to suppress the maturation of IL-1β. Finally, AA suppressed the activation of the NLRP3 inflammasome. AA significantly inhibited oxidative stress by decreasing the production of mtROS, a known NLRP3 inflammasome activator (Heid et al., 2013), thus contributing to the subsequent decreased assembly of the NLRP3 inflammasome and release of IL-1β in BV2 microglia cells (Wang et al., 2017).

Moreover, we identified MMP in BV2 microglia cells, another indicator of mitochondrial dysfunction. We found that LPS diminished MMP significantly in BV2 microglia cells. Mitochondria serve as a regulatory center of apoptosis (Susin et al., 1999). After pretreatment with AA, the MMP of LPS-induced BV2 microglia cells decreased significantly. LPS is known to increase intracellular mtROS to activate the NLRP3 inflammasome. Meanwhile, LPS can diminish MMP and incite mitochondrial dysfunction, which contributes to endogenous apoptosis.

Next, we explored intracellular mechanisms to study whether AA had protective effects on MPP+-induced DA neuron death. The death of DA neurons in the substantia nigra is central to the pathology of PD, but the neurodegenerative mechanisms have not been fully elucidated (Hirsch et al., 1988). Some studies have reported that ROS are involved in apoptosis induction following MPP+-induced neurotoxicity, and may underlie the apoptotic processes observed in PD (Di Monte et al., 1986; Kehrer and Smith, 1994). In general, the cytotoxicity of MPP+ to DA neurons was arbitrated mainly by excessive generation of ROS (Singer and Ramsay, 1990). In fact, studies have shown that AA exhibits protective effects on rotenone-induced apoptosis in SH-SY5Y cells by reducing oxidative stress, maintaining the MMP and regulating the expression of Bcl-2, Bax, and caspases (Jew et al. 2010). Furthermore, AA could also effectively offer neuroprotection against...
MPP+-induced neuronal cell loss by activating the ERK and PI3K/Akt/mTOR/GSK-3β pathways in vivo (Nataraj et al., 2017). Therefore, we verified the neuroprotective effects of AA on DA neurons. Here, MitoSOX red fluorescence suggested that MPP+-induced oxidative stress increased the accumulation of intracellular mtROS. JC-1 fluorescence indicated that MPP+ decreased MMP. Strikingly, AA had protective effects on mitochondria of DA neurons.

In summary, our data demonstrate that AA protects BV2 microglia cells against LPS-induced neuroinflammation. Pre-treatment with AA suppressed cellular cytotoxicity, inhibited the release of mtROS and protected the mitochondria from dysfunction. We also affirmed that AA had protective effects against MPP+-induced DA neuron death. This study emphasizes that AA can suppress NLRP3 inflammasome activation and protect SH-SY5Y cells from MPP+-induced damage directly in PD. In-depth mechanistic studies of signal pathways to elucidate precise protective mechanisms of AA against LPS-induced neuroinflammation in BV2 microglia cells, and the protective effects on MPP+-induced DA neuron death warrants further investigation.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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