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ORIGINAL PAPER

Curcumin exerts anti-inflammatory and antioxidative properties in 1-methyl-4-phenylpyridinium ion (MPP+)-stimulated mesencephalic astrocytes by interference with TLR4 and downstream signaling pathway

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Abstract Neuroinflammation is closely associated with the pathophysiology of neurodegenerative diseases including Parkinson's disease (PD). Recent evidence indicates that astrocytes also play pro-inflammatory roles in the central nervous system (CNS) by activation with toll-like receptor (TLR) ligands. Therefore, targeting anti-inflammation may provide a promising therapeutic strategy for PD. Curcumin, a polyphenolic compound isolated from *Curcuma longa* root, has been commonly used for the treatment of neurodegenerative diseases. However, the details of how curcumin exerts neuroprotection remain uncertain. Here, we investigated the protective effect of curcumin on 1-methyl-4-phenylpyridinium ion-(MPP+-) stimulated primary astrocytes. Our results showed that MPP+ stimulation resulted in significant production of tumor necrosis factor (TNF)-α, interleukin (IL-6), and reactive oxygen species (ROS) in primary mesencephalic astrocytes. Curcumin pretreatment decreased the levels of these pro-inflammatory cytokines while increased IL-10 expression in MPP+-stimulated astrocytes. In addition, curcumin increased the levels of antioxidant glutathione (GSH) and reduced ROS production. Our results further showed that curcumin decreased the levels of TLR4 and its downstream effectors including NF-κB, IRF3, MyD88, and TIRF that are induced by MPP+ as well as inhibited the immunoreactivity of TLR4 and morphological activation in MPP+-stimulated astrocytes. Together, data suggest that curcumin might exert a beneficial effect on neuroinflammation in the pathophysiology of PD.

Keywords Curcumin · Astrocytes · 1-Methyl-4-phenylpyridinium ion · Neuroinflammation · Oxidative stress · Toll-like receptor 4

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Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases among elderly people (Phani et al. 2012). The pathological symptoms of PD are characterized by akinesia, rest tremor, and loss of the dopaminergic neurons (DNs) in the nigra striatal (Dauer and Przedborski 2003; Noelker et al. 2013). Although the mechanisms underlying this disease remain unclear, there have been numerous studies suggesting that neuroinflammation plays a prominent role in the pathogenesis of PD (More et al. 2013; Tansey and Goldberg 2010). Neuroinflammation is triggered by activation of glial cells. The activated cells produce a variety of inflammatory mediators such as inflammatory cytokines, which in turn act on these cells and result in further expression of inflammatory cytokines (Hirsch and Hunot 2009).

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Astrocytes are the most abundant glial cell type in the brain, and they exert dual roles in regulating brain immune reactivity (Farina et al. 2007; Parpura et al. 2012). On the one hand, astrocytes possess a variety of physiological roles in brain functions, such as nutritional support for neurons and tissue repair (Rossi 2015). On the other hand, overactive astrocytes also play a pro-inflammatory role in the central nervous system (CNS) through production of a number of inflammatory mediators including cytokines, chemokines, and reactive oxygen intermediates (Farina et al. 2007; Hirsch and Hunot 2009).

Toll-like receptors (TLRs) are a class of pattern recognition receptors (PRRs) that interact with exogenous conserved components of pathogens or endogenous "danger signals" and further trigger intracellular signals (Akira et al. 2001; Tang et al. 2007; Zhang et al. 2013). In brain, TLRs are primarily present in immune cells like microglia and astrocytes (Jack et al. 2005). Astrocytes carry a series of TLRs, including TLR2, TLR3, TLR4, and TLR9 (Bowman et al. 2003; Carpentier et al. 2005). Increasing evidence indicates that TLRs play important roles in the pathologies of PD. For example, Noelker et al. showed that mice with TLR4 deficiency were less vulnerable to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication (Noelker et al. 2013). Additionally, Ma et al. showed that activation of astrocytes via TLRs exhibited neurotoxic effect to neurons in vitro (Ma et al. 2013). Therefore, we speculated that astrocytic TLRs might be involved in the neuroinflammatory conditions in PD, and proper regulation of astroglial-related inflammation may offer a potential approach for PD therapy.

Curcumin, a bioactive component primary extracted from *Curcuma longa* rhizome, is shown to exert multiple pharmacological properties, such as anti-inflammatory (Jurenka 2009), anti-oxidant (Ak and Gulcin 2008), and anti-tumor actions (Lin et al. 2007). Recently, numerous studies have shown the therapeutic potential of curcumin in neurodegenerative and neuroinflammatory diseases (Lee et al. 2013; Mansouri et al. 2012; Ray and Lahiri 2009). For example, Zhu et al. (2014) showed that curcumin attenuated acute inflammation in an experimental traumatic brain injury model. Kaur et al. (2015) demonstrated that curcumin attenuated cognitive deficits in an experimental chronic epilepsy model via inhibition of glial activation and inflammatory mediator production. Seyedzadeh et al. (2014) further revealed that curcumin exerted anti-inflammatory actions on reactive astrocytes in vitro, suggesting the potential regulatory role of curcumin in astroglial-related inflammation. Based on these findings, we now investigate the effects of curcumin on 1-methyl-4-phenylpyridinium ion (MPP$^+$)-stimulated primary mesencephalic astrocytes and depict the role of TLR4 in the inflammatory process.

Materials and methods

Cell culture

Primary astrocytes were isolated from the brain tissues of 1–3-day-old rat pups as previously described (Ma et al. 2013). Briefly, the mesencephalic tissues were minced and digested in 0.1 % trypsin for 30 min at 37 °C. After being washed with PBS, the cells were resupended in Dulbecco modified eagle medium (DMEM, Gibco, Rockville, MD, USA) containing 10 % fetal bovine serum (FBS, Hyclone, Logan, UT, USA). The cells were passaged at 6 days, and astrocytes were purified from the primary mixed cultures by three to four repetitions of trypsinization and replating. The purity of astrocytes was evaluated by immunoflurescence staining with anti-glial fibrillary acidic protein (GFAP) antibody, a specific marker of astrocytes.

Cell viability assay

The effect of curcumin on cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Sigma Co., St. Louis, MO, USA). Briefly, cells (2×10^3/well) were seeded in 96-well plates and incubated in a humidified atmosphere containing 5 % CO_2. The cells were then treated with different concentrations of curcumin (0, 1, 2, 4, 8, or 16 μM) for 48 h, followed by incubation in MTT (0.2 mg/ml) for an additional 4 h. After that, the medium was removed, and the crystal dye was dissolved in 100 μl of DMSO for each well. The absorbance was measured at 490 nm with a microplate reader (ELx800, BioTek, Winooski, VT, USA).

Real-Time PCR

Total RNA from primary astrocytes was extracted using an RNA extraction kit (Tiangen Co., Beijing, China), then converted into complementary DNA (cDNA) using Super MMLV Reverse Transcriptase (BioTeke, Beijing, China) according to the manufacturer's instructions. The expression of TLR4, MyD88, TRIF/TRAM, NF-κB, and IRF3 was determined using Exicycler™ 96 real-time (RT)-PCR machine (Bioneer, Daejeon, Korea). The sequences of all RT-PCR primers are as follows: NF-κB, 5 -CGCATTCTGA CCTTGCCTATC-3 (forward), 5 -AGTCCAGTCTC CGAGTGAAGC-3 (reverse); IRF3, 5 -TGGCTGACTT TGGCATCT-3 (forward), 5 -AATCGCAACACT TCTTTCC-3 (reverse); MyD88, 5 -TCCCACTCGCAG TTTGTT-3 (forward), 5 -TGCCTCCCAGTTCCTTTG-3 (reverse); TRAM, 5 -GGACAGACCCGAGGCAAAG-3 (forward), 5 -CCAGGCGGACCCATTTAC-3 (reverse); TLR4, 5 -GGGGGGTATTTGACACACTCTA-3 (forward), 5 -TCCTTTGGATGTCTCTATGCGA-3 (reverse); β-actin,

5 -GGAGATTACTGCCCTGGCTCCTAGC-3 (forward), 5 -GGCCGGACTCATCGTACTCCTGCTT-3 (reverse).

Measurement of cytokines

Cells were pretreated with curcumin for 30 min, followed by MPP$^+$ stimulation (800 µM) for 48 h. The concentrations of TNF-α, IL-6, and IL-10 in the supernatants were determined using enzyme-linked immunosorbent assay (ELISA) according to the products' manufacturers (Boster Bio-Engineering Co., Ltd, Wuhan, China).

Measurement of oxygen species

Reactive oxygen species (ROS) levels were determined as previously described (Morimoto et al. 2013). ROS formation was detected by monitoring a conversion of 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) to DCF according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). Briefly, single-cell suspensions were incubated with 10 µM DCF H$_2$-DA for 20 min at 37 °C. After washing three times using PBS, the cells were analyzed in a BD Accuri C6 flow cytometer (Becton, Dickinson, & Co. (BD), Franklin Lakes, NJ, USA).

Measurement of glutathione

Cells lysates were prepared by freezing and thawing method. GSH level from the supernatants was estimated using a glutathione (GSH) assay kit (Nanjing Jiancheng Institute, Nanjing, China) according to the manufacturer's instructions. GSH levels were expressed as micromoles of GSH formed per milligram of protein.

Western blotting

Cell samples were lysed using NP-40 lysis reagent (Beyotime Technology, Haimen, China), and the protein concentration was measured using a bicinchoninic acid method (Beyotime). Each amount of protein samples were separated by SDS-PAGE, then transferred onto the polyvinylidene difluoride membrane (PVDF, GE Healthcare UK, Buckinghamshire, UK). After being blocked with 5 % skim milk, the membranes were incubated in appropriate primary antibodies: anti-TLR4 (1:100, sc-293072, Santa Cruz, CA, USA), anti-MyD88 (BA2321), anti-TRIF/TRAM (BA2933-2, 1:400), anti-NF-κB (BA0610, 1:400), and anti-IRF3(BA4351-2, 1:400) (all from Boster Bio-Engineering Co., Ltd). The membranes were subsequently incubated with secondary antibodies conjugated with horseradish peroxidase (goat anti-rabbit IgG horseradish peroxidase (HRP), goat anti-mouse IgG-HRP, 1:5000, Beyotime) for 1 h. The target proteins were visualized with an enhanced ECL reagent, and the intensity of the bands was analyzed by using Gel-Pro

Analyzer software 4.0 (Media Cybernetics Inc., Rockville, MD, USA).

Immunocytochemistry

Cells cultured on a glass coverslip were washed with PBS and fixed in 4 % paraformaldehyde, then blocked with normal goat serum. After that, the cells were incubated with diluted mouse monoclonal anti-GFAP antibody and anti-TLR4 antibody, respectively (1:50 diluted, Santa Cruz Biotechnology). Then, the cells were incubated with Cy3-labeled goat anti-mouse antibody (1:100, Beyotime) at room temperature for 1 h in the dark, followed by incubation with DAPI. Cell images were visualized with a fluorescent microscope (BX53; Olympus, Tokyo, Japan).

Statistical analysis

The statistical analysis was performed using GraphPad Prism Software version 5.0 (San Diego, CA, USA). Data are expressed as the mean ± SD. Comparisons were made between the groups using one-way ANOVA, followed by Bonferroni post hoc test. A P value less than 0.05 was considered to indicate a statistically significant difference.

Results

Effect of curcumin on cell viability

The potential cytotoxicity of curcumin on primary cultured astrocytes was evaluated using an MTT assay. As shown in Fig. 1, curcumin at low concentrations (1–8 µM) did not exhibit cytotoxicity to astrocytes. However, curcumin at high concentration (16 µM) caused a significant reduction in cell
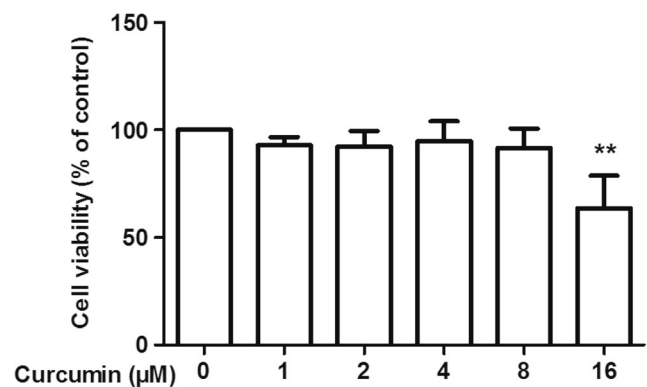

Fig. 1 Effect of curcumin on cell viability in primary astrocytes. Cells were incubated with different concentrations of curcumin (0, 1, 2, 4, 8, or 16 µM) for 48 h. Cell viability was determined by MTT assay. Data are expressed as the mean ± standard deviation. **$P < 0.01$, compared with control untreated astrocytes

viability compared to the control untreated astrocytes ($P<0.01$). Thus, we chose curcumin at a concentration of 8 μM for subsequent studies.

## Curcumin inhibited the MPP+-induced activation of primary astrocytes

Previously, we have shown that curcumin inhibited astrocyte activation in the substantia nigra and striatum in MPTP-treated mice (Yu et al. 2010). Here, we further investigated the effect of curcumin on MPP+-induced astrocytes in vitro. Glial fibrillary acidic protein, the specific marker of astrocytes, was used to detect astrocyte activation. As shown in Fig. 2, MPP+ stimulation induced a reactive state in the astrocyte, as observed by increased GFAP expression and larger cell body of astrocytes

determined by immunofluorescence. In contrast, curcumin pretreatment reversed these changes and mitigated the activation of astrocytes by MPP+. Curcumin per se had no obvious effects on the morphology of astrocytes.

Curcumin inhibited the production of pro-inflammatory cytokines in MPP+-stimulated primary astrocytes

Inflammatory cytokines are reported to be over-expressed following MPP+ insults. Thus, we investigated the effect of curcumin on inflammatory cytokine production in MPP+-stimulated astrocytes. Figure 3 shows the expression levels of TNF-α, IL-6, and IL-10 in the cell culture supernatants. MPP+ significantly increased the production of TNF-α and IL-6 compared to the control untreated astrocytes ($P<0.01$, Fig. 3a, b). Pretreatment with curcumin reduced secretion of

Fig. 2 Curcumin inhibits the MPP+-induced activation of primary astrocytes. GFAP immunostaining in primary cultured astrocytes. Curcumin reduced GFAP expression compared with the MPP+-stimulated astrocytes. Magnification, ×400, scale bars = 20 μm
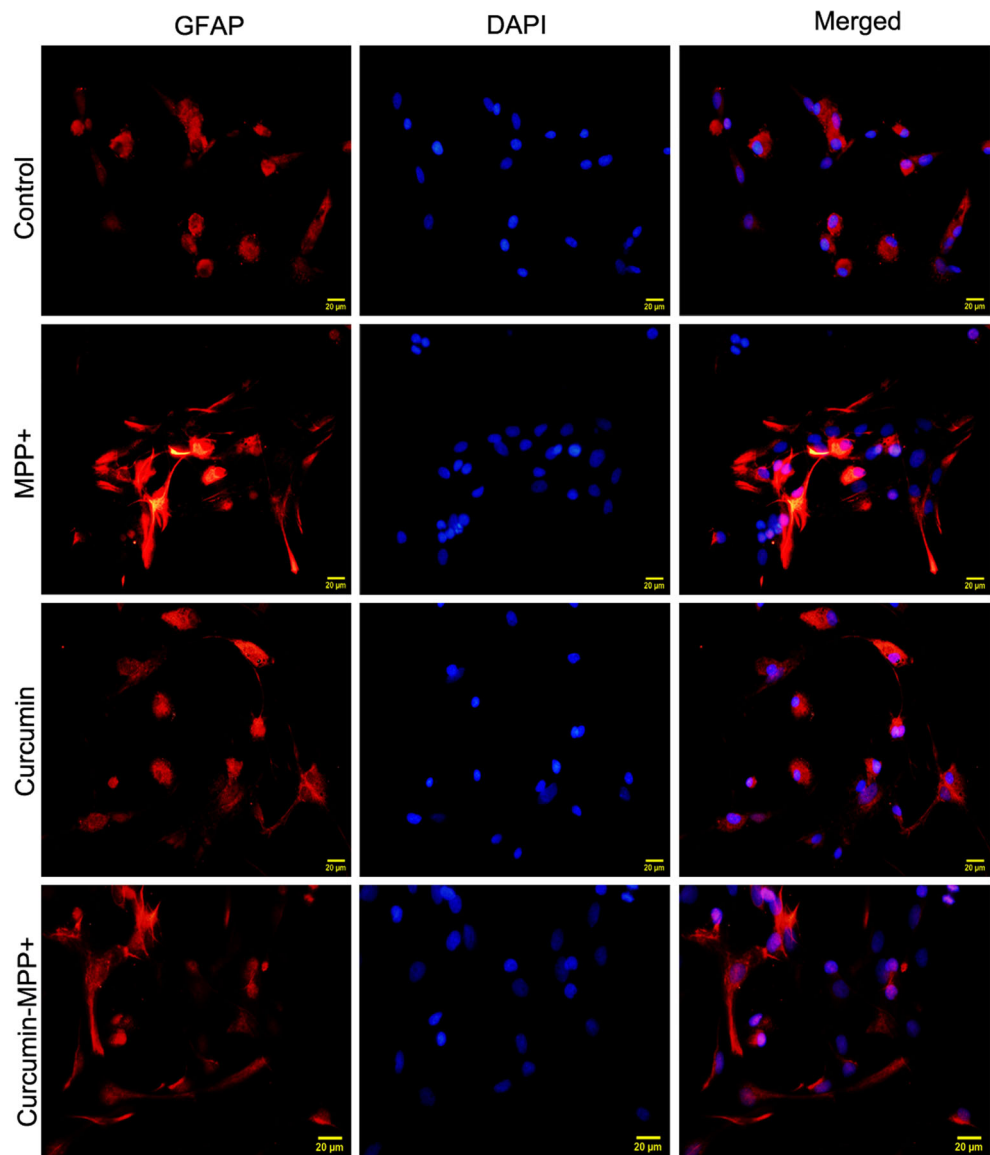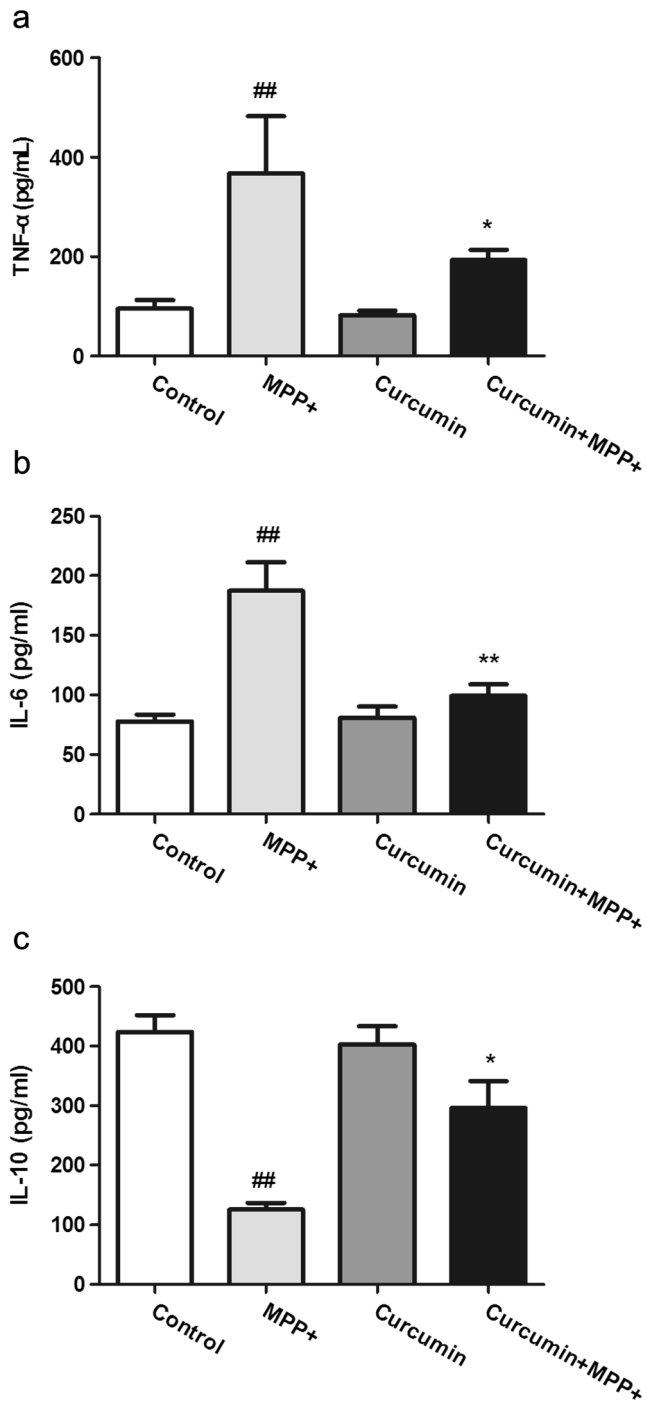
Fig. 3 Effect of curcumin on MPP+-induced cytokine production in primary astrocytes. Expression levels of **a** TNF-α, **b** IL-6, and **c** IL-10 in cell culture supernatants were measured. Curcumin downregulates TNF-a and IL-6 expression while it increased IL-10 production in MPP+-stimulated astrocytes. Data are expressed as the mean ± standard deviation. $^{##}P < 0.01$, compared with control untreated astrocytes; $*P < 0.05$, $**P < 0.01$, compared with MPP+-stimulated astrocytes

TNF-α and IL-6 ($P < 0.05$, $P < 0.01$ versus MPP+ group, respectively), while it increased the levels of IL-10 in MPP+-challenged astrocytes ($P < 0.05$, Fig. 3c). Curcumin per se did not affect the levels of TNF-α and IL-6.

Curcumin attenuated MPP+-induced oxidative mediator in astrocytes

Reactive oxygen (ROS) plays a key role in inflammation and the pathogenesis of PD. Increased ROS production overwhelms the inherent antioxidant defense like GSH (Khan et al. 2014a). Therefore, we further assessed the effect of curcumin on oxidative mediator production in MPP+-stimulated astrocytes. As shown in Fig. 4, the levels of ROS significantly increased in MPP+-challenged astrocytes compared to the control untreated astrocytes ($P < 0.01$). Curcumin effectively suppressed ROS production induced by MPP+ ($P < 0.01$, Fig. 4a). In addition, curcumin notably increased the GSH levels that were repressed by MPP+ ($P < 0.01$, Fig. 4b).

Curcumin inhibited NF-κB and IRF3 activation in MPP+-stimulated astrocytes

NF-κB and IRF3 play key roles in regulating inflammatory responses (Karin and Delhase 2000). To determine whether
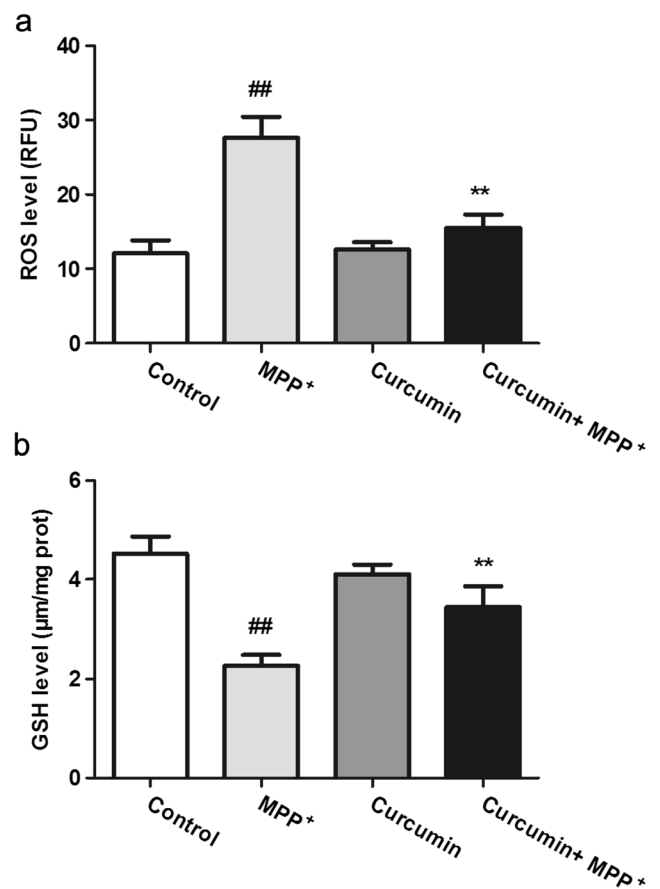

Fig. 4 Effect of curcumin on MPP+-induced oxidative stress in primary astrocytes. **a** Expression levels of ROS in astrocytes were determined by monitoring a conversion of DCF H-DA to DCF. **b** Expression levels of GSH in the medium of primary astrocytes. Data are expressed as the mean ± standard deviation. $^{##}P < 0.01$, compared with control untreated astrocytes; $**P < 0.01$, compared with MPP+-stimulated astrocytes

curcumin attenuates inflammation by affecting NF-κB and IRF3 pathways, we assess whether curcumin attenuates inflammation through the NF-κB and IRF3 pathways; the expression of NF-κB and IRF3 in astrocytes was detected by RT-PCR and Western blot analysis. As shown in Fig. 5, MPP[+] challenge significantly increased the messenger RNA (mRNA) expression of NF-κB and IRF3 ($P < 0.01$, Fig. 5a, b) and the phosphorylation of NF-κB and IRF3 (Fig. 5e, f). Furthermore, the expression of TLR4 downstream signaling components including MyD88 and TRIF were similarly up-regulated in MPP[+]-stimulated astrocytes (Fig. 5c–f). In

contrast, curcumin reversed these changes induced by MPP[+]. Together, these results indicate that curcumin attenuates astrocytic inflammation which is partially mediated by inactivation of NF-κB and IRF3.

Curcumin inhibited TLR4 expression in MPP[+]-stimulated astrocytes

Activated astrocytes via TLRs have shown neurotoxicity (Ma et al. 2013). Thus, we further determined the changes in astrocytic TLR expression after MPP[+] challenge and the effect
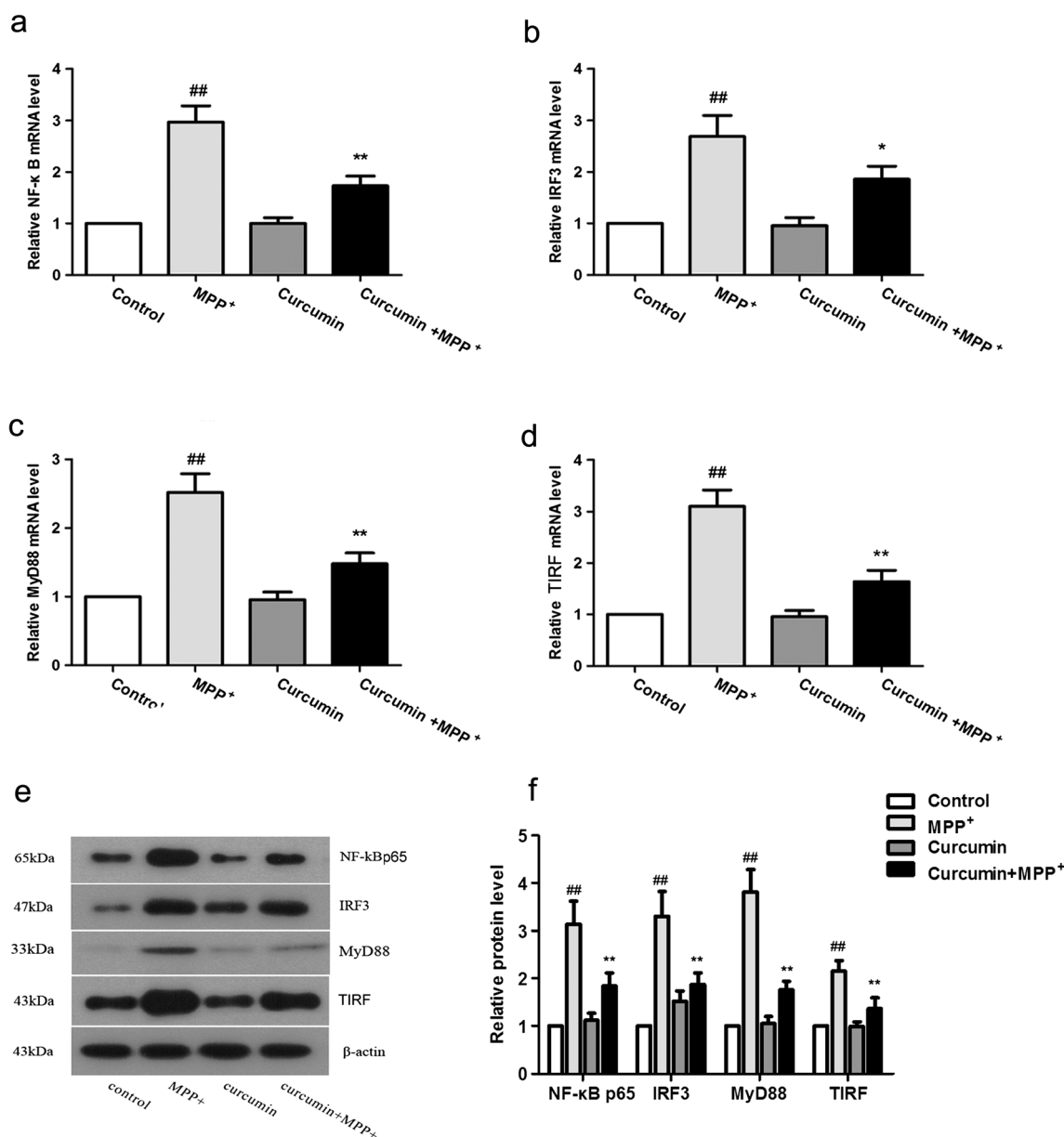

Fig. 5 Curcumin inhibits MPP[+]-induced NF-κB and IRF3 activation in primary astrocytes. Real-time PCR was performed to detect the mRNA levels of **a** NF-κB, **b** IRF3, **c** MyD88, and **d** TIRF/TRAM in primary astrocytes. **e** Western blot analysis was performed to determine the protein levels of NF-κB, IRF3, MyD88, and TIRF/TRAM. **f** Relative protein levels were quantified by a gray analysis with normalization to β-actin. Data are presented as mean ± SD; [##]$P < 0.01$, compared with control untreated astrocytes; *$P < 0.05$, **$P < 0.01$, compared with MPP[+]-stimulated astrocytes

of curcumin on TLR expression. Results from RT-PCR and Western blot analysis showed that MPP[+] exposure significantly increased the mRNA and protein levels of TLR4 in astrocytes compared to the control untreated cells. However, these elevations were repressed by curcumin pretreatment (Fig. 6a, b). Immunofluorescence results further confirmed that the MPP[+]-induced TLR4 immunoreactivity was diminished by curcumin (Fig. 6c). Together, these results indicate that the anti-inflammatory action of curcumin on MPP[+]-stimulated astrocytes is mediated by inhibition of the TLR4 signaling pathway.

Discussion

Repeated exposure to environmental toxins, such as MPTP, has been reported to cause PD symptoms (Schober 2004). MPP[+] is the active metabolite of classical parkinsonian toxin MPTP and is commonly used to investigate the alteration of cell functions relevant to PD. The toxic mechanisms of MPP[+] are demonstrated to inhibit complex I of the mitochondrial respiratory chain and induce ROS generation (Alvarez-Fischer et al. 2013; Bournival et al. 2012) as well as induce pro-inflammatory mediators of glial cells (Khan et al. 2014a;

Zhou et al. 2016). We previously showed that curcumin prevented dopaminergic neuronal death and inhibited astrocyte activation in a mouse model of MPTP-induced PD (Yu et al. 2010). In the present study, we focused on the pro-inflammatory roles of astrocytes and we specifically addressed the effects of curcumin on MPP[+]-induced primary cultured astrocytes. Our results showed that curcumin prevented the inflammatory and oxidative mediators in MPP[+]-induced mesencephalic astrocytes. Furthermore, we demonstrated for the first time that these effects were partially mediated by inhibition of TLR4 and its downstream signaling pathway.

Neuroinflammation is a central event related to PD progression. A prominent feature of neuroinflammation is characterized by glial activation, concomitant with production of pro-inflammatory mediators including cytokines, chemokines, and ROS (Hirsch and Hunot 2009; van Loo et al. 2006). Previously, glial cell activation was confirmed in the postmortem brain of PD patients and MPTP-treated animals (Damier et al. 1993; De Miranda et al. 2015). In the present study, we found that MPP[+]-stimulated astrocytes exhibited increased GFAP expression and larger cell bodies compared to the control untreated astrocytes. In addition, MPP[+] challenge led to production of TNF-α and IL-6 in

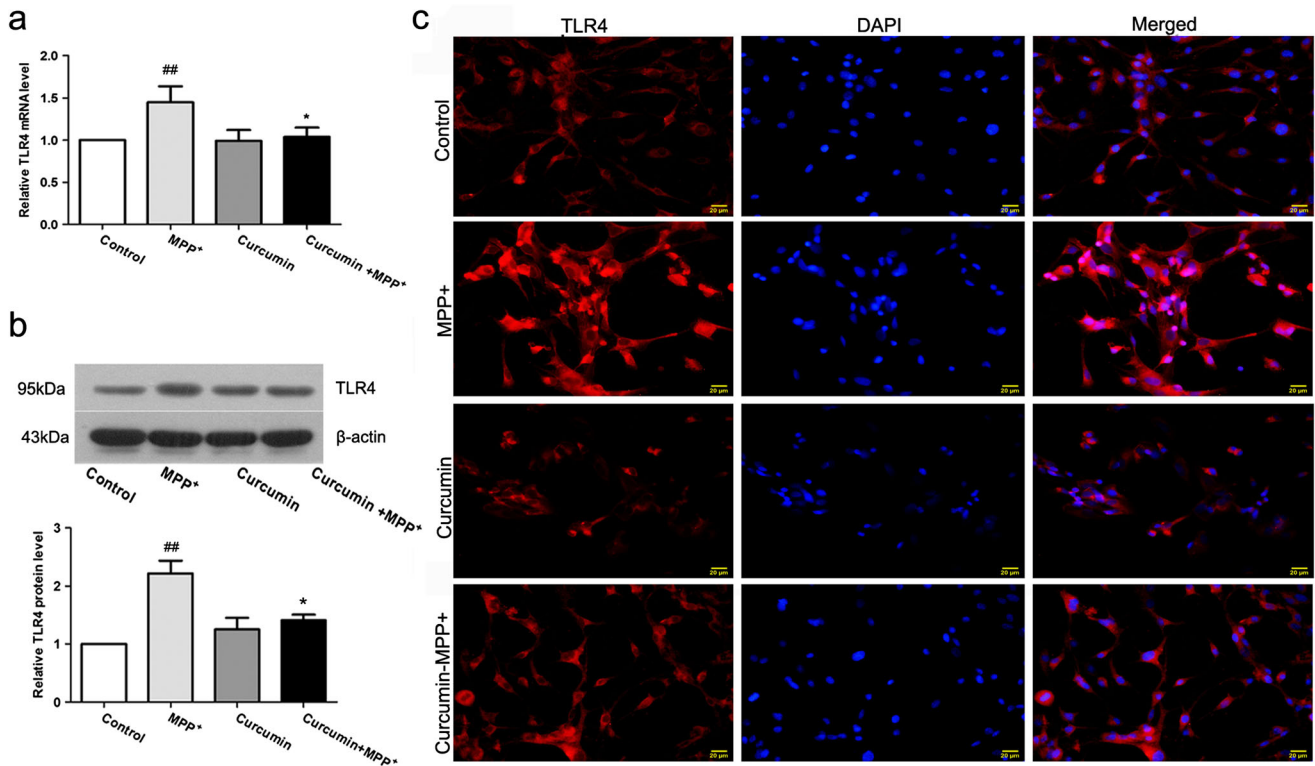Fig. 6 Curcumin attenuates the expression and immunoreactivity of TLR4 in MPP[+]-induced astrocytes. a Real-time PCR analysis was performed to determine TLR4 mRNA level of TLR4 in primary astrocytes. b The protein levels of TLR4 were determined by Western blot analysis. Relative TLR4 protein level was quantified by a gray analysis with normalization to β-actin. c The immunofluorescence of TLR4 in astrocytes was determined. TLR4 was shown in *red* and DAPI was shown in *blue*. Magnification, ×400, *scale bars* = 20 μm. Data are presented as mean ± SD; [##]$P < 0.01$, compared with control untreated astrocytes; *$P < 0.05$, compared with MPP[+]-stimulated astrocytes (color figure online)

astrocytes. However, curcumin pretreatment inhibited the MPP$^+$-induced activation of astrocytes and decreased the expression levels of TNF-α and IL-6. Our results indicate that curcumin can diminish MPP$^+$-induced inflammation and the activation of astrocytes, and our results were consistent with an earlier study showing that curcumin exerted an anti-inflammatory effect in lipopolysaccharide (LPS)-induced human astrocyte cell line (Seyedzadeh et al. 2014).

ROS production plays a key role in inflammatory process and the pathogenesis of PD. Previously, Wong et al. showed that the mesencephalic astrocytes were more prone to produce ROS than were other regional astrocytes after MPP$^+$ stimulation (Wong et al. 1999). In the present study, we found that MPP$^+$-treated astrocytes exhibited significant increase in ROS production. However, this change was reversed by curcumin pretreatment. Furthermore, curcumin restored the level of antioxidant GSH that is decreased by MPP$^+$. Together, these results suggest that curcumin can mitigate oxidative stress in MPP$^+$-induced mesencephalic astrocytes.

TLRs are a large class of immune molecules mediating innate immune responses through conserved signaling pathways (Akira et al. 2001; Beutler et al. 2003). There are two major downstream pathways in TLR signaling: the MyD88-dependent pathway and the MyD88-independent pathway (or TRIF pathway) (Kumar et al. 2009). MyD88 is a key adaptor protein required by all TLRs except TLR3 (Akira et al. 2006). The MyD88-dependent pathway induces production of various pro-inflammatory mediators via NF-κB or MAPK activation (Kagan and Medzhitov 2006). In contrast, the MyD88-independent pathway is controlled by the signal transduction intermediates like TRIF, which trigger production of type I interferon via IRF3 activation (Akira and Sato 2003). Previous studies have shown that MPP$^+$-induced cytotoxicity is related to NF-κB activation (Khan et al. 2014a; Liu et al. 2010) and ROS generation (Khan et al. 2014b). ROS is supposed to be one of the causes leading to TLR4 upregulation (Wei et al. 2015). In the present study, we showed that MPP$^+$ stimulation increased the expression levels of NF-κB and MyD88 in astrocytes. Moreover, we also found that the expression levels of IRF3 and TRIF were upregulated in MPP$^+$-stimulated astrocytes. Curcumin significantly inhibited the activation of NF-κB and IRF3 and decreased the levels of TLR4 and its downstream effectors including MyD88 and TIRF that were induced by MPP$^+$. Together, these results suggest that the anti-inflammatory action of curcumin is mediated via an inhibition of TLR4 and its downstream signaling pathway. Previously, curcumin was shown to have a number of molecular targets involved in inflammation (Ghosh et al. 2015). Curcumin could inhibit LPS-induced NFκB activation through inhibiting IKKβ (Pan et al. 2000). Youn et al. (2006) further showed that curcumin attenuated inflammatory responses through inhibiting homodimerization of TLR4. Here, we showed that curcumin exerted anti-inflammatory

action in MPP$^+$-induced astrocytes through downregulating TLR4 and its downstream signaling. Our results were consistent with these findings.

In conclusion, our results demonstrated that curcumin decreased the production of pro-inflammatory and oxidative mediators (TNF-α, IL-6, and ROS) in MPP$^+$-stimulated mesencephalic astrocytes. The protective mechanism was partially through inhibiting both MyD88-dependent and TRIF-dependent pathways in TLR4 signaling. Thus, TLR4-mediated astrocytic inflammation could be a potential target for PD therapy and curcumin administration could be a potential agent in the inflammatory pathology of PD.

Compliance with ethical standards

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