Ellagic acid produces neuroprotection against LPS-induced dopamine neurotoxicity via the inhibition of microglial NLRP3 inflammasome activation

Xuemei He
Zunyi Medical University
Qiuyu Yang
Zunyi Medical University
Jingyi He
Zunyi Medical University
Shuo Sheng
Zunyi Medical University
Jingjie Li
Zunyi Medical University
Daidi Li
Zunyi Medical University
Guoqing Wang
Zunyi Medical University
Feng Zhang (zhangfengzmc@163.com)
Zunyi Medical University

Research

Keywords: Parkinson's disease, Microglia, Inflammasome, Ellagic acid, Neuroprotection

Posted Date: February 25th, 2020

DOI: https://doi.org/10.21203/rs.2.24392/v1

Abstract

Background: Neuroinflammation plays a crucial role in the pathological process of Parkinson's disease (PD). Nod-like receptor protein 3 (NLRP3) inflammasome was highly located in microglia and involved in the process of neuroinflammation. Activation of NLRP3 inflammasome has been confirmed to contribute to the progression of PD. Thus, inhibition of NLRP3 inflammasomes activation could be an important breakthrough point in PD drug therapy. Ellagic acid (EA) is a natural polyphenol that has been widely found in soft fruits, nuts and other plant tissues with various anti-inflammatory and anti-oxidant properties. However, the mechanisms underlying EA-mediated anti-inflammatory and neuroprotection have not been fully elucidated.

Methods: In this study, lipopolysaccharide (LPS)-induced rat dopamine (DA) neuronal damage model was performed to determine the effects of EA on the protection of DA neurons. Furthermore, DA neuron MN9D cell line and microglia BV-2 cell line were employed to explore whether EA-mediated neuroprotection was through an NLRP3-dependent mechanism.

Results: EA ameliorated LPS-induced DA neuronal loss in rat substantia nigra. Furtherly, inhibition of microglial NLRP3 inflammasome signaling activation was involved in EA-generated neuroprotection, as evidenced by the following observations. First, EA reduced NLRP3 inflammasome signaling activation in microglia and the subsequent pro-inflammatory cytokines excretion. In addition, EA-mediated anti-neuroinflammation and further DA neuroprotection from LPS-induced neurotoxicity was not shown upon microglial NLRP3 siRNA treatment.

Conclusions: This study demonstrated EA has a profound effect on protecting DA neurons against LPS-induced neurotoxicity via the suppression of microglial NLRP3 inflammasome signaling activation.

Introduction

Parkinson's disease (PD) is a chronic progressive neurodegenerative disease characterized by a deep selective loss of dopamine (DA) neurons in the substantia nigra (SN) [1]. Clinical manifestations include static tremor, slow movement, postural instability, stiffness and other motor disorders [2]. So far, only a few drugs have been available for PD treatment and most of them just relieve symptoms, not prevent the death of DA neurons [3].

It was recognized that age-related excessive oxidative stress led to DA auto-oxidation, α-synuclein accumulation, and glial cells activation [4], which are the main causes of neuroinflammation [5]. Furtherly, neuroinflammation, characterized by microglia activation and infiltrating T cells at sites of neuronal injury, was considered to be a prominent contributor to the pathogenesis of progressive PD [6]. Microglia are natural immune barriers in the immune system. While stimulated by external conditions, such as brain damage, inflammation and pathogens, microglia could be activated and secrete a range of pro-inflammatory factors [7]. These factors led to the damage of adjacent DA neurons, which in turn elicited more microglia to reactivate in response, forming a self-propelling cycle [8]. The inflammasome involving

NLRP3 (NLR family, pyrin domain containing 3)-CASP1 (caspase 1) can regulate the maturation of IL-1β and IL-18 [9, 10]. Additionally, nod-like receptor protein 3 (NLRP3) inflammasome was highly located in microglia and involved in the process of neuroinflammation [11]. Activation of NLRP3 inflammasomes had been discerned to play an important role in the progression of neurodegenerative diseases [12]. Therefore, inhibition of NLRP3 inflammasomes activation could be an important breakthrough point in PD drug therapy.

Ellagic acid (2, 3, 7, 8-tetrahydroxybenzopyrano [5, 4, 3-cde] benzopyran-5-10-dione, EA) is a natural polyphenol that has been widely found in soft fruits, nuts and other plant tissues. EA exhibits a number of pharmacological activities, such as anti-oxidant and anti-inflammatory effects [13]. Recent studies confirmed EA exerted neuroprotection against aging, ischemic stroke and neurodegenerative disorders. For example, EA generated neuroprotection and improved cognitive dysfunctions in sporadic Alzheimer's disease animal model [14]. In addition, EA was indicated to confer neuroprotection against ischemic stroke [15]. However, the underlying mechanisms remain unclear. In this study, rat substantia nigral stereotaxic single injection of lipopolysaccharide (LPS)-elicited DA neuronal loss was performed to investigate EA-exerted neuroprotection and the underlying mechanisms as well. These findings would provide evidence for the future application of EA on PD treatment.

Materials And Methods

Reagents

EA (purity>95%), LPS (Escherichia coli O111:B4) and 6-hydroxydopamine (6-OHDA) were obtained from Sigma Aldrich (St. Lewis, CA, USA). Enzyme-linked Immunosorbant assay (ELISA) kits for TNF-α, IL-1β and IL-18 were bought from Elabscience Biotechnology Co., Ltd (Wuhan, China). MTT assay kit was from Beijing Solarbio science and Technology Co., Ltd. (Beijing, China). The small interfering RNA (siRNA) against NLRP3 was purchased from GenePharma (Shanghai, China). Anti-CR3 complement receptor (OX-42 Catalog No. Ab1211) and tyrosine hydroxylase (TH, Catalog No. Ab113) antibodies were bought from Abcam (Cambridge, MA, USA). Anti-caspase-1 (Catalog No. 22915-1-AP), ionized calcium-binding adapter molecule-1 (Iba-1, Catalog No. 10904-1-AP), TNF-α (Catalog No. 17590-1-AP), IL-1β (Catalog No. 66737-1-Ig), IL-18 (Catalog No. 10663-1-AP), β-actin (Catalog. No. 20536-1-AP), rabbit IgG (Catalog No. SA00001-2) and mouse IgG (Catalog No. SA00001-1) antibodies were purchased from Proteintech Group (Chicago, IL, USA). Anti-NLRP3 (Catalog No. orb101128) antibody was purchased from Biorbyt (Cambridge, United Kingdom).

Animal and Treatment

Male Wistar rats (200–250 g, 8–10 weeks old) were bought from the Experimental Animal Center in the Third Military Medical University. All experimental procedures were carried out in accordance with Chinese Guidelines of Animal Care and Welfare and this study received an approval from the Animal Care and Use Committee of Zunyi Medical University (Zunyi, China). Rats were acclimated to their environment for 1 week before the experiments. All the animals were randomly allocated to five experimental groups with

six rats in each group: control, EA alone (50 mg/kg), LPS, LPS+EA (10 mg/kg) and LPS+EA (50 mg/kg). With anesthetized by 7% chloral hydrate (0.5 ml/100 g, v/w), rats received a single LPS (10 μg in 5 μl PBS) unilateral injection into the SN pars compacts followed by the coordinates 5.2 mm posterior to bregma, 1.9 mm lateral to the midline, and 8.0 mm ventral to the surface of the skull [16]. Daily intragastric injection of EA (10 and 50 mg/kg) was administrated for 7 consecutive days after LPS injection. Control animals were injected with a single PBS into SN pars compacts. Afterwards, animals were sacrificed and the biochemical analysis was performed.

Rotarod Test

Rotarod test was performed to study the muscular coordination. It contained cylindrical arrangement of the thin steel rods with two parts by compartmentalization to permit the detection of two rats at the same time. Prior to the start of the test, rats were allowed to remain stationary at 0 rpm. Then, the rotational speed was steadily increased to 10 rpm in 30 s interval till rats fell off the rungs. The duration time each rat stayed on the rod was recorded and calculated for analyzing the behavior changes of rats [17].

Immunohistochemical Analysis and Cell Counting in the SN

Rat brains were cut with a horizontal sliding microtome into 35 μm transverse free-floating sections and immunostained with the corresponding antibodies. Briefly, brain slices were incubated with 0.3% Triton X-100 and blocked with goat serum. Subsequently, brain slices were incubated with anti-TH (1:500), OX-42 (1:800), and NLRP3 (1:800) antibodies at 4 °C overnight, respectively [18]. Digital images of TH-positive neurons, OX-42-positive microglia and NLRP3-positive inflammasome in midbrain SN were acquired by an Olympus microscope (Olympus, Tokyo, Japan). Quantification of TH-positive neurons was determined through visually counting the number of TH-positive neuronal cell bodies blindly by two investigators and results were analyzed from the average. The mean value for SN TH-positive neuronal numbers was then deduced by averaging the counts of 6 sections for each rat. Representative fluorescence images of OX-42 and NLRP3 inflammasome were obtained and the fluorescence intensity was calculated using ipwin32 software.

Cell Culture and Treatment

Mouse microglial BV-2 cell lines were obtained from China Center for Type Culture Collection (Wuhan, China). Cultures were maintained in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in the humidified atmosphere of 5% CO_2 and 95% air [19]. DA neuron MN9D cell lines were purchased from the Cell Culture Center in the Institute of Basic Medical Sciences of Chinese Academy of Medical Sciences (Beijing, China). MN9D cells were cultured in DMEM medium with 10% FBS and 1% penicillin-streptomycin on an atmosphere with 5% CO_2 at 37 °C in the humidified atmosphere of 5% CO_2 and 95% air [20].

MTT Assay

Cell viability was evaluated by MTT assay. BV-2 and MN9D cells were cultured in 1×10^5/well in 96-well plates for 24 h. Afterwards, cells were treated with different concentrations of EA for 30 min followed by LPS (100 ng/ml) or 6-OHDA (100 μM) treatment for another 24 h and then incubated with MTT solution (5 g/l) for 4 h. Formazan crystals in the cells were solubilized using 200 μl dimethyl sulfoxide (DMSO) and the absorbance was detected by an automated microplate reader within 490 nm wavelength [21].

Western Blot Analysis

Total protein content was extracted from rat midbrain tissue and BV-2 cells using a lysis buffer containing protease inhibitors. Protein levels were quantified by BCA assay [22]. Protein (10 μg) from each sample was subjected to SDS-PAGE gel under the reduced conditions. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk for 2 h at room temperature and then incubated overnight at 4 °C with the primary antibodies: Iba-1 (1:800), NLRP3 (1:800), caspase-1 (1:800), TNF-α (1:1000)IL-1β (1:500), IL-18 (1:800) and β-actin (1:2000). Next, the membranes were incubated for 1 h with a horseradish-peroxidase-conjugated anti-mouse IgG antibody or anti-rabbit IgG at 1:2000 dilution. The blot films were developed with enhanced ECL Reagent.

ELISA

The levels of TNF-α, IL-1β, and IL-18 were measured by ELISA according to the manufacturer's instructions. The microplate reader was used to measure the absorbance at 450 nm [23].

Immunofluorescence Staining

Activated microglia were identified with an anti-OX-42 antibody. Cells were fixed with paraformaldehyde (4%) for 30 min. Later, cells were permeabilized using triton X-100 (0.3%) for 15 min. Then, cells were blocked using goat serum blocking solution for 60 min at 37 °C. Thereafter, cells were incubated with 1:800 dilution of anti-OX-42 antibody overnight at 4 °C. Following overnight incubation, cells were incubated in dark for 30 min with goat anti-rabbit secondary antibody (1:1000) or goat anti-mouse secondary antibody (1:1000). Cells were also counterstained with DAPI for 5 min [24]. After rinsing cells with PBS, representative fluorescence images were obtained using EVOS® Floid® Cell imaging station. The fluorescence intensity was calculated by using ipwin32 software.

RNA Transfection

BV2 cells were cultured and seeded in a 6-well plate at a density of 1×10^5 cells/ml. The transfection of siRNA was performed complying with the manufacturer's protocol. Briefly, NLRP3 siRNA (2 μl) was diluted into 18 μl of transfection medium. GP-siRNA-Mate plus (180 μl) was used to transfect with siRNAs dilution (20 μl), then made up 2 ml with MEM medium. After 6 h of transfection, the transfection solution was removed. Cells were rinsed with PBS and replaced with MEM medium containing 2% FBS. Then, cells were treated with EA for 30 min followed by LPS (100 ng/ml) stimulation for another 24 h [25]. The gene

sequences were as follows: Sense 5’-UUC UCC GAA CGU GUC ACG UTT-3; antisense 5’-ACG UGA CAC GUU CGG AGA ATT-3’.

Statistical Analysis

Results were indicated as mean ± standard error of the mean (SEM). Statistical significance was analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Upon ANOVA demonstrating the significant differences, pairwise comparison between means was evaluated by Bonferroni's *post hoc* test with correction. A value of $p<0.05$ was considered statistically significant.

Results

EA attenuated LPS-induced DA neuronal damage in the SN *in vivo*

Neuroprotective effects of EA on LPS-induced DA neuronal damage were investigated in rats. As shown in **Figure 1A**, LPS reduced the time rat stayed on the rod, compared with control group. However, EA attenuated LPS-caused decrease in the time rat remained on the rod. To further confirm EA-mediated DA neuroprotection, TH-positive neuronal number and TH protein expression were determined. As shown in **Figure 1B**, EA ameliorated LPS-induced decrease in TH protein expression. Consistent with TH protein detection results, TH-positive neuronal counting showed EA protected against LPS-induced DA neuronal loss (**Figure 1C**).

EA ameliorated LPS-elicited activation of microglia and NLRP3 inflammasome signaling *in vivo*

Next, the effects of EA on microglia and NLRP3 inflammasome activation were investigated. To verify the connection between NLRP3 inflammasome and microglia, the immunofluorescence double calibration site was conducted. As shown in **Figure 2A,** the NLRP3 inflammasome was activated and located in activated microglia. EA attenuated LPS-induced activation of NLRP3 inflammasome in microglia. Also, EA inhibited Iba-1 protein expression induced by LPS (**Figure 2B**). In addition, EA suppressed LPS-induced activation of NLRP3 inflammasome signaling (**figure 2C**) and pro-inflammatory cytokines (1L-1β, TNF-α and 1L-18) protein expressions (**figure 2D).**

EA had no direct neuroprotective effects on DA neurons

To further confirm whether EA produced direct neuroprotective actions on DA neurons, the effects of EA on 6-OHDA -induced DA neuronal damage *in vitro* were determined. First, as shown in **Figure 3A**, 6-OHDA induced neurotoxicity was not attenuated by EA treatment in MN9D cell-enriched cultures. Next, in TH-positive neurons counting analysis (**Figure 3B and C**), 6-OHDA caused TH-positive neuronal loss and EA didn’t protect from 6-OHDA-induced neuronal damage. Similar results were indicated in TH protein detection shown in **Figure 3D**. These results demonstrated that EA didn’t generate direct neuroprotection on DA neurons.

EA inhibited microglial NLRP3 inflammasome activation *in vitro*

The effects of EA on microglia and NLRP3 inflammasome signaling activation were further confirmed *in vitro*. First, BV-2 cells were employed to examine the effects of EA on LPS-induced microglial activation. As shown in **Figure 4A**, immunofluorescence staining assay indicated that EA reduced LPS-induced microglia activation. Also, EA decreased LPS-induced higher protein expression of Iba-1 (**Figure 4B**). Then, EA inhibited the activation of microglial NLRP3 inflammasome signaling induced by LPS (**Figure 4B**). In addition, EA eliminated LPS-induced production of pro-inflammatory factors, such as TNF-α, IL-1β and IL-18 in the culture medium (**Figure 4C**).

NLRP3 inflammasome signaling inactivation was involved in EA-mediated anti-inflammatory properties

To investigate the role of NLRP3 inflammasome signaling in EA-mediated anti-neuroinflammation, NLRP3 siRNA was performed in BV-2 cell cultures. First, as shown in **Figure 5A,** NLRP3 siRNA was transfected into microglia cells and the successful transfection with NLRP3 siRNA was evaluated by NLRP3 protein level in BV-2 cells. Furthermore, NLRP3 siRNA and EA inhibited LPS-induced NLRP3 inflammasome signaling activation, respectively. However, no significant difference of NLRP3, caspase-1 and pro-caspase-1 protein expressions between LPS+EA and LPS+EA+NLRP3 siRNA groups was discerned (**Figure 5B**). In addition, the effects of EA on pro-inflammatory factors excretion with NLRP3 siRNA administration were measured. In parallel with NLRP3 inflammasome signaling analysis, EA didn't reduce LPS-induced release of pro-inflammatory factors again after NLRP3 siRNA treatment (**Figure 5C**). These observations indicated EA attenuated microglia-induced neuroinflammation via the inhibition of NLRP3 inflammasome signaling activation.

EA targeted microglial NLRP3 inflammasome to produce DA neuroprotection

Since microglia were the target of EA-generated DA neuroprotection, whether this neuroprotection resulted from inhibiting microglial NLRP3 inflammasome activation was then explored. As shown in **Figure 6**, compared with MCM (LPS) group, both MCM (LPS+NLRP3 siRNA) and MCM (LPS+EA) protected against MCM (LPS)-induced neurotoxicity evidenced by cell viability and TH protein expression detection, whereas no significant difference of neuroprotection between these two groups was exhibited. Furtherly, MCM (LPS+ NLRP3 siRNA+EA) didn't exert more DA neuroprotection against MCM (LPS)-caused neuronal injury than MCM (LPS+NLRP3 siRNA) or MCM (LPS+EA) treatment, either.

Discussion

The present study aimed to investigate the neuroprotective actions of EA on LPS-induced DA neuronal loss and evaluate the role of microglia in this neuroprotection. Results indicated that EA protected DA neurons against LPS-induced neurotoxicity in SN. Furtherly, inhibition of microglial NLRP3 inflammasome signaling activation was involved in EA-generated neuroprotection, as evidenced by the following observations. First, EA reduced NLRP3 inflammasome signaling activation in microglia and the subsequent pro-inflammatory cytokines excretion. In addition, EA-mediated anti-neuroinflammation and

further DA neuroprotection from LPS-induced neurotoxicity was not shown upon microglial NLRP3 siRNA treatment. Taken together, EA conferred neuroprotection against LPS-induced DA neuronal damage via the inhibition of microglial NLRP3 inflammasome signaling activation.

Neuroinflammation is considered to be a most common feature of the aging brain and neurodegenerative disorders including PD. It is primarily mediated by activated glial cells and accompanied by the secretion of pro-inflammatory mediators [26]. As the first defense of immune surveillance, microglia readily become activated and predominately participate in inflammatory response. In addition, astroglia could amplify microglia-mediated neuroinflammation and result in the feedback loop of neuroinflammatory reactions [27]. In this regard, understanding the molecular mechanisms of PD can be found by investigating the microglial neuroinflammation [28]. Therefore, to target microglia-derived pro-inflammatory cytokines might offer promising therapeutic approaches for PD management. Inflammasomes are multi-protein complexes responsible for intracellular sensors of environmental and cellular stress [29]. Inflammasomes consist of NLRP family, the signaling adapter apoptosis associated speck-like protein containing a caspase recruitment domain (ASC) and pro-inflammatory precursor pro-caspase-1 [30]. In addition, NLRP3 inflammasome is particularly located in microglia and involved in the response of neuroinflammation [11]. Upon cellular stress, assembly of NLRP3 inflammasome triggers caspase-1 activation and further caspase-1-mediated production of IL-1β and IL-18, thereby initiating neuroinflammation [31]. In PD patient brains, the NLRP3 inflammasome is potentially activated by insoluble a-synuclein aggregates and oxidative stress [32]. However, deficiency of NLRP3 inflammasome attenuates motor dysfunction and DA neurodegeneration in PD mouse model [33]. Thus, inhibition of NLRP3 inflammasome activation might be beneficial for PD intervention. In the present study, EA-mediated DA neuroprotection was discerned in neuron-microglia co-cultures but not in neuron-enriched cultures, implying that microglia were at least essential for EA-generated neuroprotection. Moreover, we found EA reduced pro-inflammatory factors production induced by LPS. Furtherly, EA-inhibited microglial activation and subsequent pro-inflammatory factors release was attributed to the inhibition of microglial NLRP3 inflammasome activation. This finding was revealed by the following observations: 1) EA inhibited microglial NLRP3 and pro-caspase-1 activation and IL-1β production. 2) EA could not further suppress LPS-induced pro- inflammatory factors release and produce DA neuroprotection after neuron-microglia co-cultures treated by NLRP3 siRNA.

To date, current PD therapy is focused on the symptoms control and fails to delay the progressive neurodegenerative process. Actually, various side-effects of the available drugs present huge challenges for long-term application. Therefore, more potential therapeutic candidates are urgently essential for halting the progression of PD. Recent studies demonstrated inhibition of neuroinflammation would attenuate DA neurodegeneration. Thus, anti-inflammatory agents might open new avenues for PD treatment. However, the low success of translating promising anti-inflammatory candidates from animal studies to clinical trials was indicated. Therefore, an urgent approach for novel anti-inflammatory alternatives design was prompted [29]. Recent studies have confirmed EA could cross blood-brain barrier [34]. Here, the present study demonstrated that EA protected from LPS-induced DA neurotoxicity and modulation of microglial NLRP3 inflammasome signaling activation was revealed to participate in this

neuroprotection. However, the current study on EA only stays in the animal experiments, and the follow-up hopes to be used as a clinical drug application for the next research.

Conclusions

This study demonstrated EA has a profound effect on protecting DA neurons against LPS-induced neurotoxicity via the suppression of microglial NLRP3 inflammasome signaling activation. These findings suggest EA might be a potential benefit for PD treatment.

Abbreviations

6-OHDA: 6-hydroxydopamine; AD: Alzheimer's disease; PD: Parkinson's disease; SN: Substantia nigra; DA: Dopamine; LPS: Lipopolysaccharide; NLRP3: Nod-like receptor protein 3; CASP1: Cysteinyl aspartate-specific proteases-1; EA: Ellagic acid; FBS: Fetal bovine serum; MEM: Minimum essential medium; IL-1β: Interleukin-1β; IL-18: Interleukin-18; TNF-α: Tumor necrosis factor-α; ELISA: Enzyme-linked Immunosorbant assay; siRNA: Small interfering RNA; OX-42: Anti-CR3 complement receptor; PBS: phosphate buffer; DMSO: dimethyl sulfoxide; PVDF: polyvinylidene fluoride; Iba-1: Ionized calcium-binding adapter molecule-1; TH: Tyrosine hydroxylase; MCM: Microglia-conditioned Medium

Declarations

Acknowledgements

Not applicable

Funding

This study was supported by the National Natural Science Foundation of China (No. 81760658), the foundation for High-level Innovative Talents of Guizhou Province (No. 20164027), Shijingshan's Tutor Studio of Pharmacology (No. GZS-201607), the Innovation Research Group project of Education Department of Guizhou Province (No. 2016038), the foundation for Excellent Young Talents of Zunyi Medical University (No.201603) and Master Start Foundation of Zunyi Medical University (No. F-898).

Availability of data and materials

The datasets used and/or analyzed during the current study were available from the corresponding author on the reasonable request.

Authors' contributions

FZ conceived and designed the experiments. All the authors participated in the experiment performance and data analysis. FZ, SS and XMH wrote, revised and checked the article. All authors revised and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were carried out in accordance with Chinese Guidelines of Animal Care and Welfare and this study received an approval from the Animal Care and Use Committee of Zunyi Medical University (Zunyi, China).

Consent for publication

Not applicable.

Competing interests

The authors declared no conflicts of interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. Zhang, Y., V.L. Dawson, and T.M. Dawson, *Oxidative stress and genetics in the pathogenesis of Parkinson's disease.* Neurobiol Dis. 2000;**7**:240-50.
2. Collier, T.J., N.M. Kanaan, and J.H. Kordower, *Aging and Parkinson's disease: Different sides of the same coin?* Mov Disord. 2017;**32**:983-990.
3. Lees, A.J., J. Hardy, and T. Revesz, *Parkinson's disease.* Lancet. 2009;**373**:2055-66.
4. Raichur, A., S. Vali, and F. Gorin, *Dynamic modeling of alpha-synuclein aggregation for the sporadic and genetic forms of Parkinson's disease.* Neuroscience. 2006;**142**:859-70.
5. Qian, L., P.M. Flood, and J.S. Hong, *Neuroinflammation is a key player in Parkinson's disease and a prime target for therapy.* J Neural Transm (Vienna). 2010;**117**:971-9.
6. Hansson, E., *Long-term pain, neuroinflammation and glial activation.* Scand J Pain. 2010;**1**:67-72.
7. Zhang, F., J. Liu, and J.S. Shi, *Anti-inflammatory activities of resveratrol in the brain: role of resveratrol in microglial activation.* Eur J Pharmacol. 2010;**636**:1-7.
8. Dauer, W. and S. Przedborski, *Parkinson's disease: mechanisms and models.* Neuron. 2003;**39**:889-909.
9. Franchi, L., R. Munoz-Planillo, and G. Nunez, *Sensing and reacting to microbes through the inflammasomes.* Nat Immunol. 2012;**13**:325-32.
10. He, Y., H. Hara, and G. Nunez, *Mechanism and Regulation of NLRP3 Inflammasome Activation.* Trends Biochem Sci. 2016;**41**:1012-1021.
11. Heneka, M.T., et al., *NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice.* Nature. 2013;**493**:674-8.

12. Freeman, L.C. and J.P. Ting, *The pathogenic role of the inflammasome in neurodegenerative diseases.* J Neurochem. 2016;**136 Suppl 1**:29-38.
13. Firdaus, F., et al., *Ellagic acid attenuates arsenic induced neuro-inflammation and mitochondrial dysfunction associated apoptosis.* Toxicol Rep. 2018;**5**:411-417.
14. Moura, F.A., et al., *Lipoic Acid: its antioxidant and anti-inflammatory role and clinical applications.* Curr Top Med Chem. 2015;**15**:458-83.
15. Liu, Q.S., et al., *Ellagic acid protects against neuron damage in ischemic stroke through regulating the ratio of Bcl-2/Bax expression.* Appl Physiol Nutr Metab. 2017;**42**:855-860.
16. Latchoumycandane, C., et al., *Dopaminergic neurotoxicant 6-OHDA induces oxidative damage through proteolytic activation of PKCdelta in cell culture and animal models of Parkinson's disease.* Toxicol Appl Pharmacol. 2011;**256**:314-23.
17. Wang, T., et al., *Hydroxysafflor Yellow A Improves Motor Dysfunction in the Rotenone-Induced Mice Model of Parkinson's Disease.* Neurochem Res. 2017;**42**:1325-1332.
18. Zhang, F., et al., *Resveratrol protects dopamine neurons against lipopolysaccharide-induced neurotoxicity through its anti-inflammatory actions.* Mol Pharmacol. 2010;**78**:466-77.
19. Song, X.M., et al., *[Inhibitory effects of acteoside on LPS-induced inflammatory response on BV-2 microglial cells].* Zhongguo Zhong Yao Za Zhi. 2016; **41**:2506-2510.
20. Dong, Y., et al., *Amperometric measurements of catecholamine release from single vesicles in MN9D cells.* J Neurochem. 2008;**107**:1589-95.
21. van Meerloo, J., G.J. Kaspers, and J. Cloos, *Cell sensitivity assays: the MTT assay.* Methods Mol Biol. 2011;**731**:237-45.
22. Hirano, S., *Western blot analysis.* Methods Mol Biol. 2012;**926**:87-97.
23. Zheng, Y., et al., *Icariin targets Nrf2 signaling to inhibit microglia-mediated neuroinflammation.* Int Immunopharmacol. 2019;**73**:304-311.
24. Donaldson, J.G., *Immunofluorescence Staining.* Curr Protoc Cell Biol. 2015;**69**:4.3.1-7.
25. Chen, C., et al., *Naringenin Produces Neuroprotection Against LPS-Induced Dopamine Neurotoxicity via the Inhibition of Microglial NLRP3 Inflammasome Activation.* Front Immunol. 2019;**10**:936.
26. Shao, W., et al., *Suppression of neuroinflammation by astrocytic dopamine D2 receptors via alphaB-crystallin.* Nature. 2013;**494**:90-94.
27. Jo, M., et al., *Astrocytic Orosomucoid-2 Modulates Microglial Activation and Neuroinflammation.* J Neurosci. 2017;**37**:2878-2894.
28. Wang, G.Q., et al., *Icariin Reduces Dopaminergic Neuronal Loss and Microglia-Mediated Inflammation in Vivo and in Vitro.* Front Mol Neurosci. 2017;**10**:441.
29. Yan, Y., et al., *Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome.* Cell. 2015;**160**:62-73.
30. Hu, Z.L., et al., *Kir6.1/K-ATP channel on astrocytes protects against dopaminergic neurodegeneration in the MPTP mouse model of Parkinson's disease via promoting mitophagy.* Brain Behav Immun.

2019;**81**:509-522.

31. Di Virgilio, F., *The therapeutic potential of modifying inflammasomes and NOD-like receptors.* Pharmacol Rev. 2013;**65**:872-905.
32. Gordon, R., et al., *Inflammasome inhibition prevents alpha-synuclein pathology and dopaminergic neurodegeneration in mice.* Sci Transl Med. 2018;**10**.
33. Lee, E., et al., *MPTP-driven NLRP3 inflammasome activation in microglia plays a central role in dopaminergic neurodegeneration.* Cell Death Differ. 2019;**26**:213-228.
34. Yan, L.L., et al., *Method Development and Validation for Pharmacokinetic and Tissue Distributions ofEllagic Acid Using Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS)*. Molecules. 2014; **19**: 18923–18935

Figures

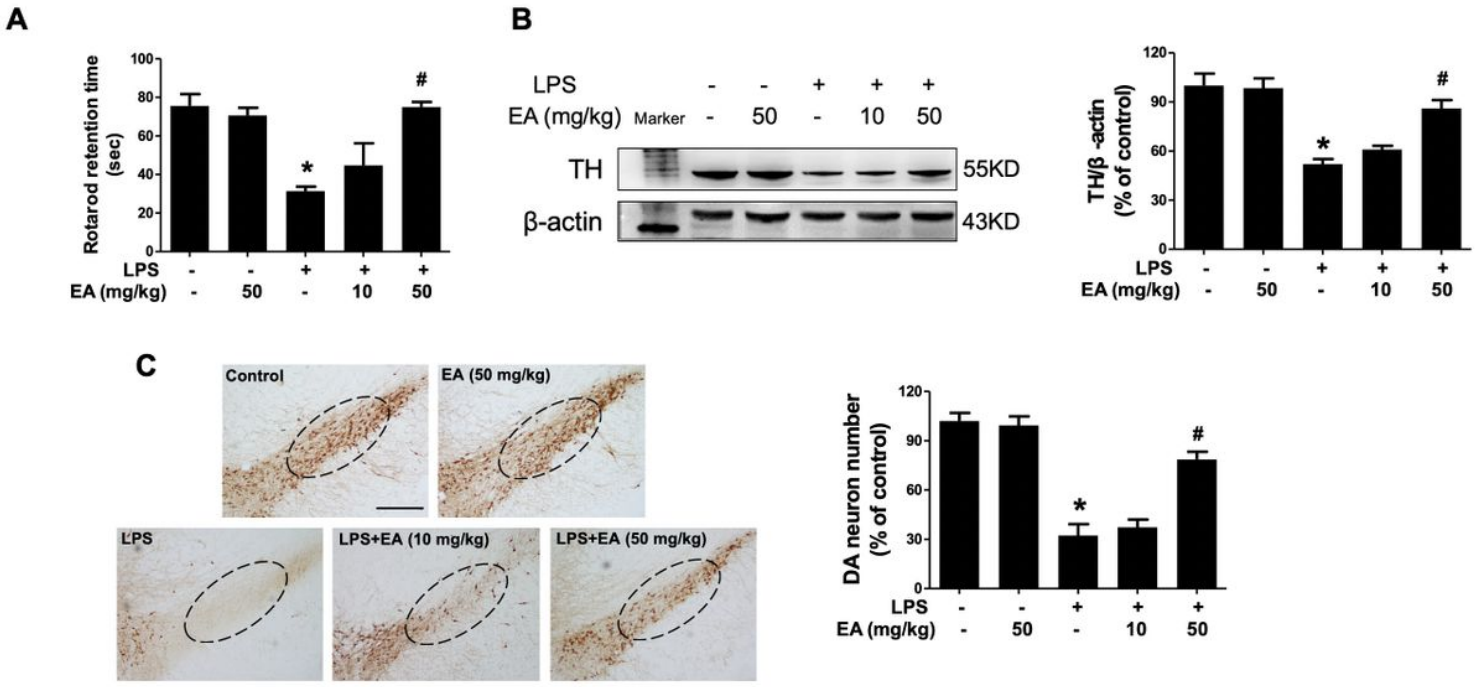

Figure 1

EA attenuated LPS-induced DA neuronal damage in the SN in vivo. Rats were intragastrically given EA (50 mg/kg) for 7 consecutive days. Rat behavior changes were analyzed by rotarod test (A). TH protein expression in rat midbrain was tested by western blot assay (B). Brain sections were immunostained with an anti-TH antibody (C), and the number of TH-positive neurons in the SN was counted. The “ellipse” presented the area of SN. Scale bar = 200 μm. Data were mean ± SEM from 6 rats. *p<0.05 compared with control group; #p<0.05 compared with LPS group.

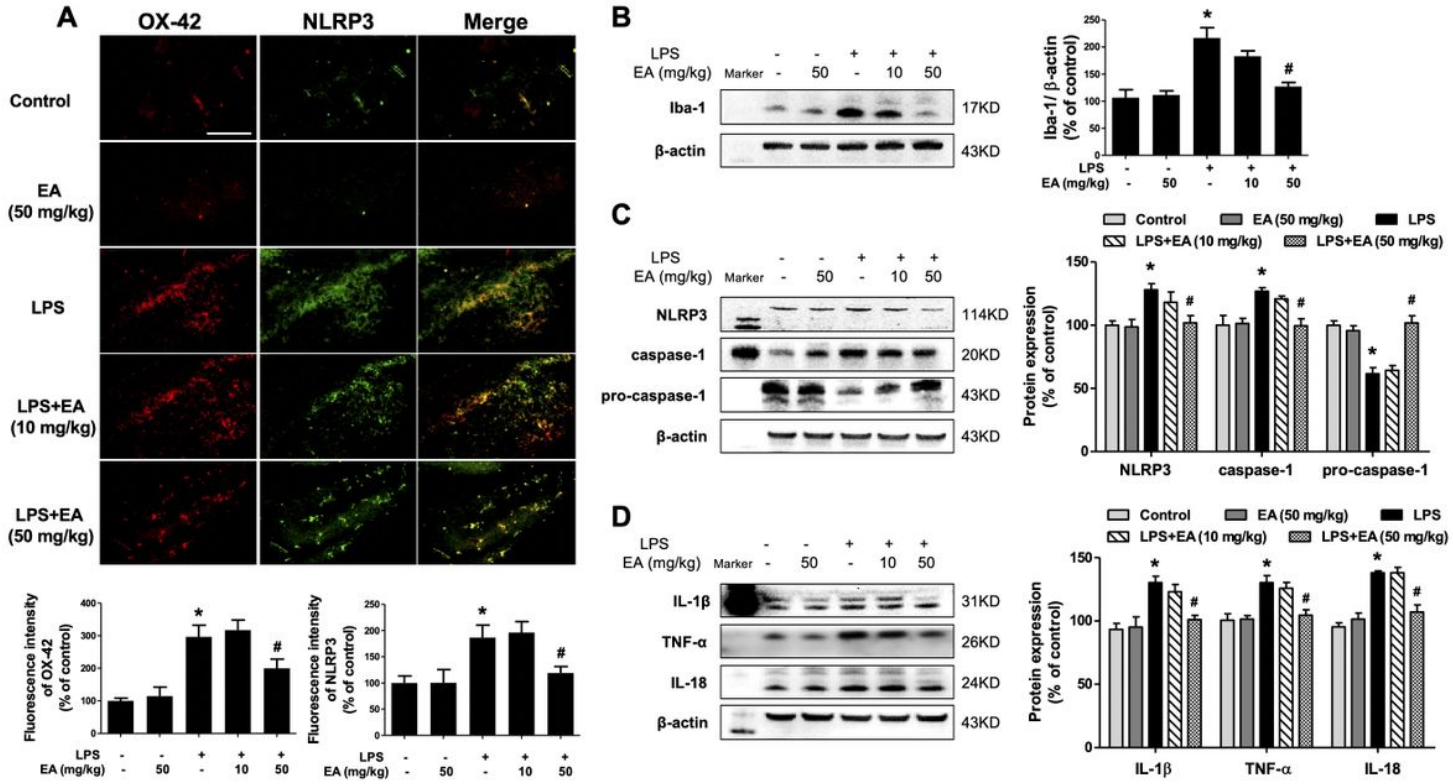

Figure 2

EA ameliorated LPS-elicited activation of microglia and NLRP3 inflammasome signaling in vivo. Rat brains were collected and stained by double-immunofluorescence with anti-NLRP3 and anti-OX-42 antibodies (green fluorescence represented NLRP3 inflammasome and red fluorescence represented microglia) (A). The protein expressions of Iba-1 (B), NLRP3, caspase-1 and pro-caspase-1 (C) and TNF-α, IL-1β and IL-18 (D) in rat midbrain were determined via western blot assay. Data were the mean ± SEM from 6 rats and expressed as a percentage of the control group. *p<0.05 compared with control group; #p<0.05 compared with LPS group.

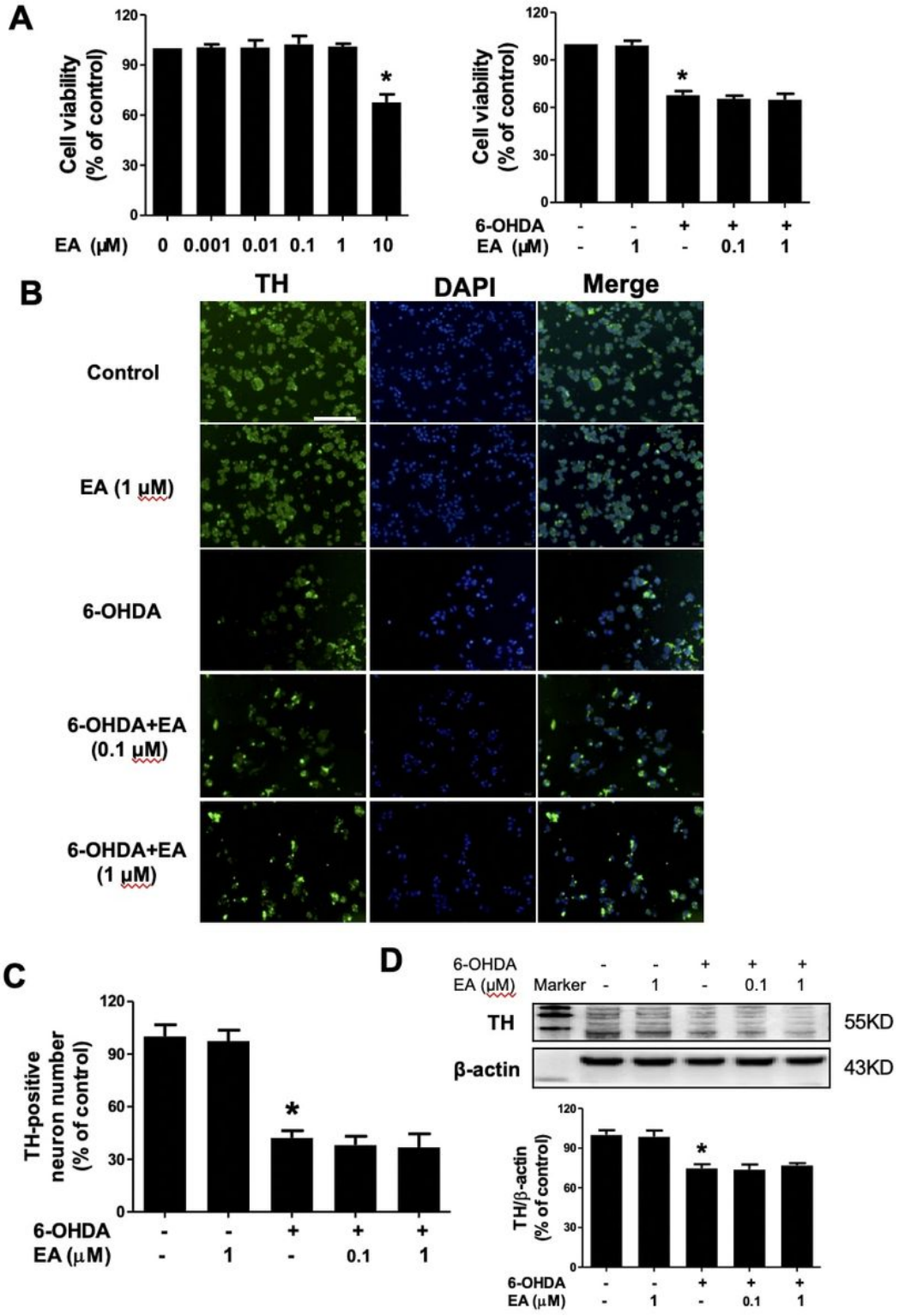

Figure 3

EA had no direct neuroprotective effects on DA neurons. MN9D cells were treated with EA (0.1 and 1 μM) for 30 min and then incubated with 6-OHDA (100 μM) for 24 h. Cell viability was determined by MTT assay (A). 6-OHDA-induced MN9D cell damage was evaluated by immunostaining (B) and cell counting (C). Scale bar = 100 μm. The protein expression of TH was detected by western blot assay (C). Data were

the mean ± SEM from three independent experiments performed in triplicate. *p<0.05 compared with control cultures; #p<0.05 compared with 6-OHDA-treated cultures.

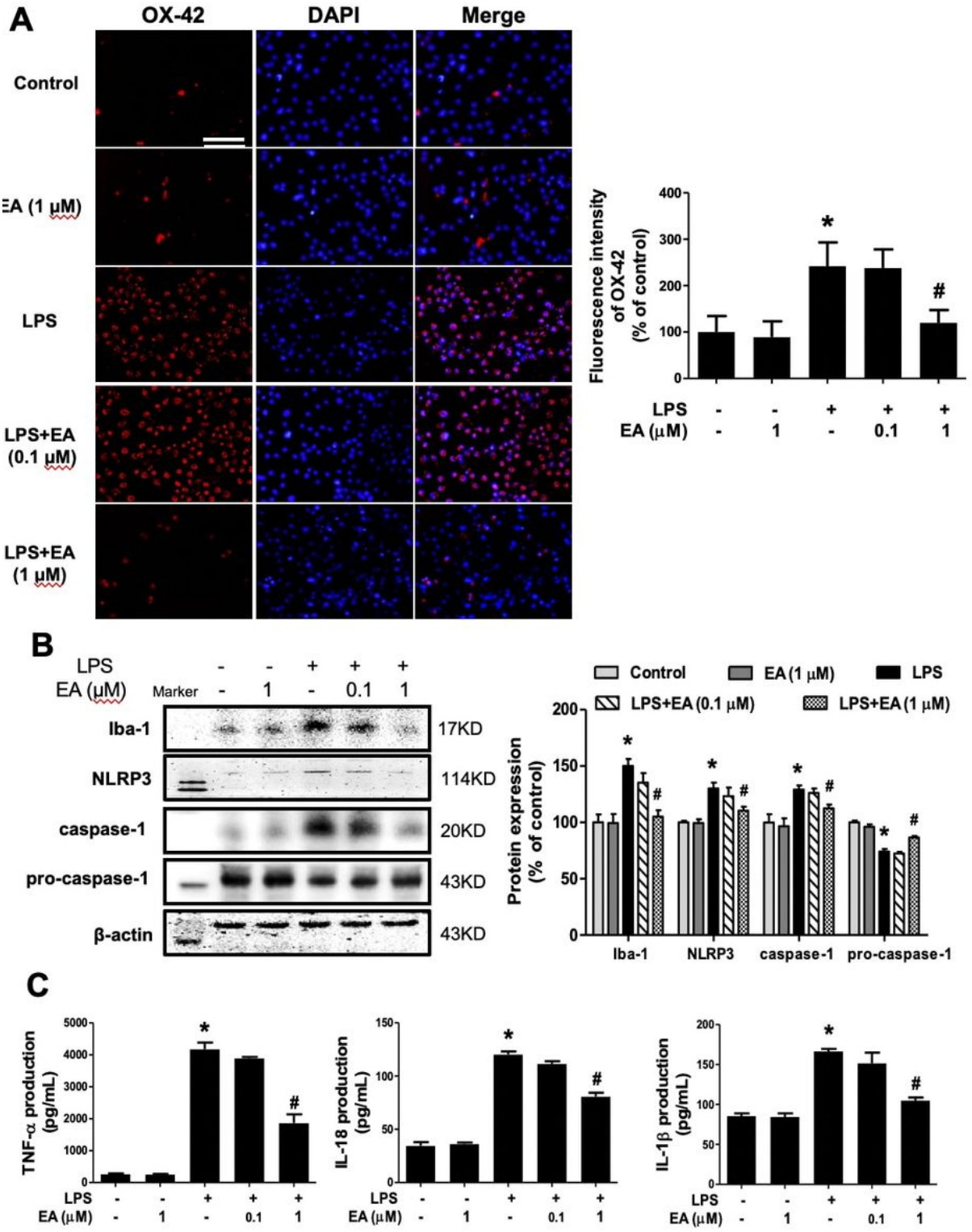

Figure 4

EA inhibited microglial NLRP3 inflammasome activation in vitro. BV2 cells were treated with EA (0.1 and 1 μM) for 30 min and then incubated with LPS (100 ng/ml) for 24 h. Microglial activation was evaluated by immunostaining (A) with an anti-OX-42 antibody and quantitated by western blot analysis with an

anti-Iba-1antibody (B). Scale bar = 100 μm. The effects of EA on NLRP3 inflammasome signaling activation in BV-2 cells were detected via western blotting (B). The ratio of densitometry values of Iba-1, NLRP3, caspase-1 and pro-caspase-1 with β-actin were analyzed and normalized to each respective control cultures. The release of pro-inflammatory factors, such as TNF-α、IL-1β, and IL-18, in BV-2 cell culture medium was measured by ELISA (C). Data were the mean ± SEM from three independent experiments performed in triplicate. *p<0.05 compared with control cultures; #p<0.05 compared with LPS-treated cultures.

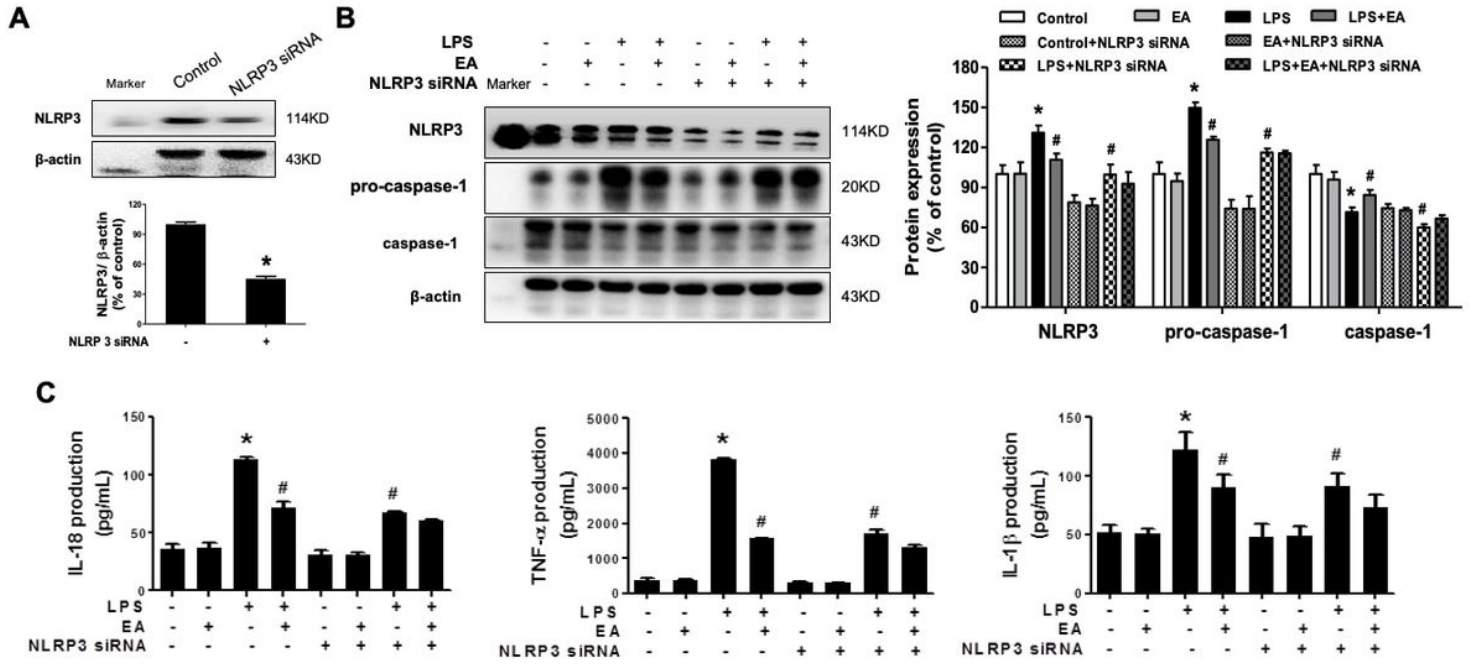

Figure 5

NLRP3 inflammasome signaling inactivation was involved in EA-mediated anti-inflammatory properties. BV-2 cells were treated with NLRP3 siRNA (40 nmol/L). After 6 h of transfection, the transfection solution was removed and cells were rinsed with PBS. The silence efficiency was assessed via NLRP3 protein expression detection (A). Moreover, BV-2 cells were treated with EA (1 μM) in the presence of NLRP3-siRNA and then exposed to LPS for 24 h. The protein expressions of Iba-1, NLRP3, caspase-1 and pro-caspase-1 in BV-2 cells were detected via western blot assay (B). The levels of TNF-α, IL-1β and IL-18 in culture medium were measured by ELISA (C). Data were the mean ± SEM from three independent experiments performed in triplicate. *p<0.05 compared with control cultures; #p<0.05 compared with LPS-treated cultures.

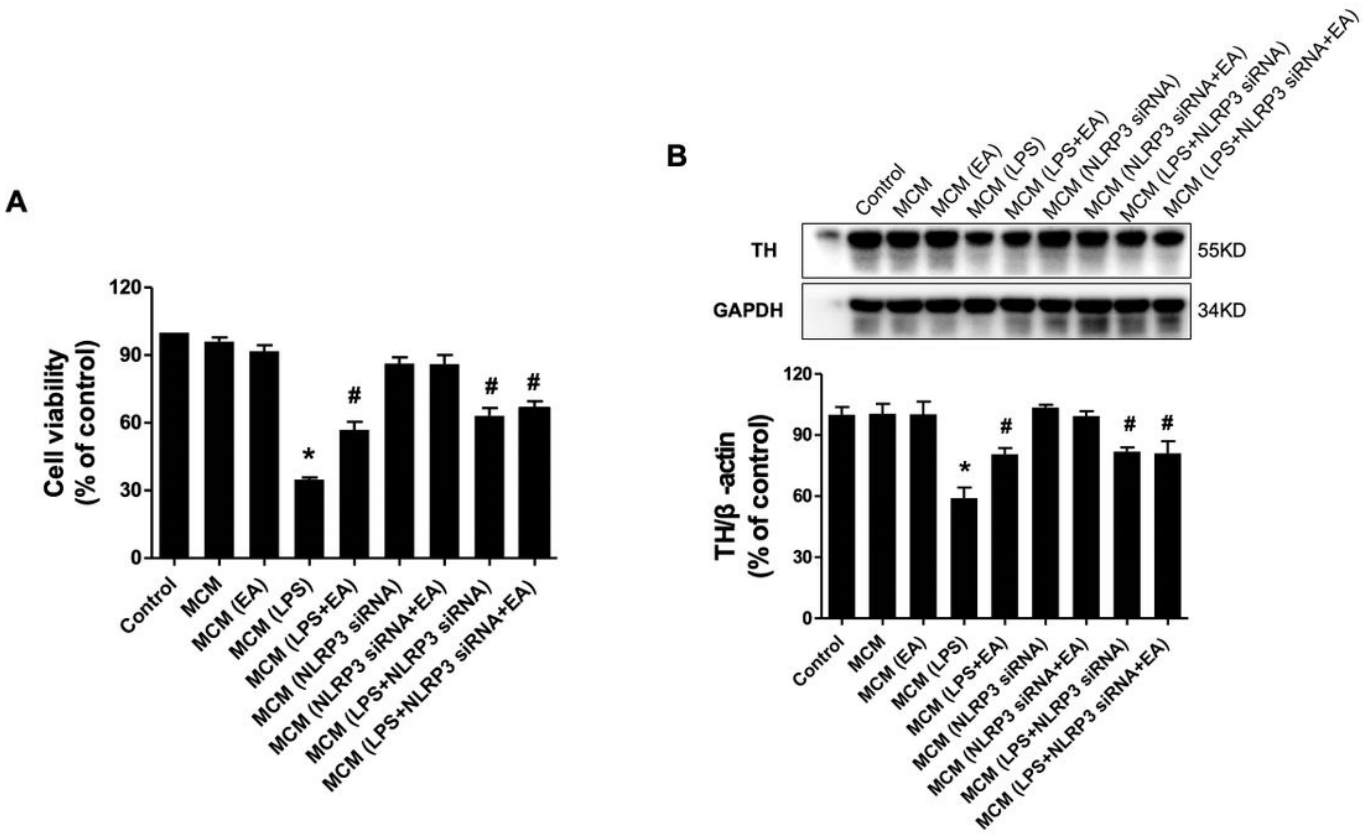

Figure 6

EA targeted microglial NLRP3 inflammasome to produce DA neuroprotection. Microglia-conditioned medium (MCM) prepared from BV-2 cell cultures with administration of EA [MCM (EA)], LPS [MCM (LPS)], LPS + EA [MCM (LPS + EA)], NLRP3 siRNA [MCM (NLRP3 siRNA)], NLRP3 siRNA + EA [MCM (NLRP3 siRNA +EA)], NLRP3 siRNA + LPS [MCM (NLRP3 siRNA + LPS)] , LPS + NLRP3 siRNA + EA [MCM (LPS + NLRP3 siRNA + EA)] were harvested and added to MN9D cells incubated for 24 h. MN9D cell viability was determined by MTT assay (A). TH protein expression was tested by western blot assay (B). Data were the mean ± SEM from three independent experiments performed in triplicate. *p<0.05 compared with control cultures; #p<0.05 compared with MCM (LPS)-treated cultures.