Research Article

trans-Cinnamaldehyde Inhibits Microglial Activation and Improves Neuronal Survival against Neuroinflammation in BV2 Microglial Cells with Lipopolysaccharide Stimulation

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Received 8 May 2017; Revised 3 September 2017; Accepted 19 September 2017; Published 22 October 2017

Academic Editor: Eman Al-Sayed

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Background. Microglial activation contributes to neuroinflammation and neuronal damage in neurodegenerative disorders including Alzheimer's and Parkinson's diseases. It has been suggested that neurodegenerative disorders may be improved if neuroinflammation can be controlled. trans-cinnamaldehyde (TCA) isolated from the stem bark of Cinnamomum cassia possesses potent anti-inflammatory capability; we thus tested whether TCA presents neuroprotective effects on improving neuronal survival by inhibiting neuroinflammatory responses in BV2 microglial cells. Results. To determine the molecular mechanism behind TCA-mediated neuroprotective effects, we assessed the effects of TCA on lipopolysaccharide (LPS) - induced proinflammatory responses in BV2 microglial cells. While LPS potently induced the production and expression upregulation of proinflammatory mediators, including NO, iNOS, COX-2, IL-1β, and TNF-α, TCA pretreatment significantly inhibited LPS-induced production of NO and expression of iNOS, COX-2, and IL-1β and recovered the morphological changes in BV2 cells. TCA markedly attenuated microglial activation and neuroinflammation by blocking nuclear factor kappa B (NF-κB) signaling pathway. With the aid of microglia and neuron coculture system, we showed that TCA greatly reduced LPS-elicited neuronal death and exerted neuroprotective effects. Conclusions. Our results suggest that TCA, a natural product, has the potential of being used as a therapeutic agent against neuroinflammation for ameliorating neurodegenerative disorders.

1. Introduction

Neuroinflammation is a critical component in both acute and chronic neurodegenerative disorders, exemplified by Alzheimer's disease (AD), Parkinson's disease (PD), ischemia, and traumatic brain injury (TBI) [1–3]. A major characteristic of brain inflammation is microglial activation that accompanies the neurodegenerative process [4]. Microglia, the resident macrophages in the brain, possess properties particularly suitable to mediate cellular inflammatory responses [5]. Microglia are activated in response to brain injury and exposure to lipopolysaccharide (LPS), interferon gamma (IFN-γ), or β-amyloid [6, 7]. Activated microglia are linked to the release of a number of proinflammatory mediators including nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), chemokines, complements, excitatory amino acids, and reactive oxygen species (ROS) that are thought to contribute to neuronal death, damage, and functional deterioration [8, 9]. Recent studies suggest that nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways including p38, extracellular signal-regulated kinase (ERK), and
c-Jun N-terminal kinase (JNK) are involved in the process of inflammation associated with microglial activation. Since microglia-mediated neurotoxicity is a crucial molecular event involved in initiation and progression of neurodegenerative disorders [10], inhibition of microglial activation may thus be a potential therapeutic approach against neuroinflammatory and neurodegenerative disorders [11, 12].

Cinnamomum cassia (C. cassia) has been used to treating dyspepsia, gastritis, blood circulation disturbances, and inflammatory disease in China for thousand years. It has been shown to provide a significantly protective effect against glutamate-induced neuronal death [13]. trans-cinnamaldehyde (TCA), a natural product, is a major bioactive component isolated from the stem bark of C. cassia [14–16] and has been reported to possess antitumor, antipyretic, antimicrobial, antidiabetic, and antimutagenic properties [17–20]. Importantly, TCA also has potent anti-inflammatory activity in aging rats, endothelial cells, and monocytes/macrophages [21–23]. Cinnamon and its main constituents including TCA have also been shown to inhibit neuroinflammation in LPS-stimulated BV2 microglial cells [24]. TCAs anti-inflammatory activity is likely to be associated with its suppressive role in toll-like receptor 4- (TLR4-) mediated signaling [25, 26] and is probably mediated by targeting multiple molecular mechanisms because it can inhibit age-related inflammatory NF-κB activation via the NIK/IKK, ERK, and p38 MAPK pathways in aging rats [21]. These results strongly suggested that TCA may represent as an effective anti-inflammatory drug to deter neurodegenerative processes. However, the molecular mechanisms of TCA for being used to protect neuronal damage under neuroinflammation remain unclear.

The objective of this study is to investigate the potential of using TCA to block neuroinflammation and to improve neuronal survival under neuroinflammation. Using LPS-stimulated microglia as a model of activated microglia, our results showed that TCA inhibited the production of NO and IL-1β and expression of iNOS and COX-2 by suppressing activation of NF-κB and granted neuroprotective effects evidenced by attenuating microglial neurotoxicity in microglia/neuron coculture system. This study suggests that TCA, a natural product, should be seriously considered as a potential therapeutic agent for a variety of neuroinflammatory and neurodegenerative diseases.

2. Materials and Methods

2.1. Chemicals and Reagents. Bacterial lipopolysaccharide (LPS) from E. coli serotype O111:B4, trans-cinnamaldehyde (TCA), and JSH-23 were purchased from Sigma-Aldrich (St. Louis, MO, USA). LPS was dissolved in sterile saline. Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute medium (RPMI), and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA). All other reagents were purchased from Sigma-Aldrich unless otherwise described.

2.2. Cell Culture. The immortalized BV2 murine microglial cell line and rat pheochromocytoma PC12 neuronal cell line were obtained from the Cell Culture Center of the Chinese Academy of Medical Sciences (China). BV2 cells were maintained at DMEM supplemented with 10% heat-inactivated FBS at 37°C in a humidified atmosphere containing 5% CO2. PC12 cells were cultured in RPMI supplemented with 10% FBS and 20% horse serum. The growth medium was replenished every third day until confluence. In all experiments, cells were treated with TCA for the indicated times before LPS stimulation.

2.3. MTT Cell Viability Assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was used to determine cell viability. Briefly, BV2 cells (2 × 10^4 cells/well) were plated in 96-well plates and then subjected to various treatment. To determine cell viability, 0.5 mg/ml MTT solution was added to cells for 4 h. Formed MTT formazan was solubilized with DMSO and quantified at 540 nm using a microplate reader (Synergy 2, BioTek Instruments, Inc., Winooski, VT, USA) and results are expressed as the percentage of cells in treated groups over the untreated Control. Each assay was carried out in triplicate.

2.4. Nitrite Quantification. BV2 cells were plated at 2 × 10^4 cells/well in 96-well plates. Cells were stimulated with LPS (0.1 μg/ml) for 24 h after pretreatment of varying concentrations of TCA. Amount of nitrite in cell culture media was analyzed by Griess reaction as previously described [27] as used to assess NO production. Briefly, 50 μl of cell supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide in water and 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid) and incubated at room temperature for 10 min followed by measuring the absorbance at 550 nm with the aid of a microplate reader. The data are the representative of three or more independent experiments.

2.5. Western Blot Analysis. To prepare lysates, BV2 cells were cultured in the presence or absence of LPS (0.1 μg/ml) or LPS + TCA (10 μM) for varying times and then lysed in ice cold RIPA buffer [150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 2 mM sodium orthovanadate (Na3VO4), and 1 mM sodium fluoride (NaF)], supplemented 1 mM phenylmethylsulfonyl fluoride (PMSF), and inhibitors of protease and phosphatase (10 μg/ml each of aprotinin, leupeptin, and pepstatin A). The nuclear proteins were then extracted using Nuclear Extraction Reagents kit (Keygen BioTECH, Nanjing, Jiangsu, China). An aliquot of 20 μg of cytosol and nuclear protein was electrophoresed on 10% and 12% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS/PAGE) gels and then transferred to a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) followed by incubation with primary antibodies. Primary antibodies used for this studies are anti-iNOS (Cell Signaling Technology, Danvers, MA, USA), anti-COX-2 (CST), anti-NF-κBp65 (CST), anti-p-IκBα (Santa Cruz Biotechnology, Dallas, Texas, USA), anti-IκBα (Santa Cruz), anti-β-actin (CST), and anti-Histone 3.
The intensities of protein bands were quantified by Image Quant software (Tanon, Shanghai, China). The relative protein level was normalized to β-actin or Histone 3.

2.6. Quantitative RT-PCR (qRT-PCR). BV2 cells (2 × 10^6 cells/well in 6-well plate) used for qRT-PCR analysis were stimulated with LPS (0.1 µg/ml) in the presence or absence of TCA (10 µM) for varying times. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and reverse transcribed using PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio Inc. Otsu, Shiga, Japan) followed by qRT-PCR. The primer sequences were included in the following:

iNOS (Forward, 5'-CACCTTGGAGTTCACCAA-GT-3'; Reverse, 5'-ACCACCTGTACCTGGGATGC-3')

IL-1β (Forward, 5'-CAGGCCAGCAGTACACT-CA-3'; Reverse, 5'-AGCTCATATGGTCCGACAG-3')

TNF-α (Forward, 5'-GAACCTGCAAGAGGCACT-3'; Reverse, 5'-AGGGTCTGGGCCATAGACT-3')

COX-2 (Forward 5'-GTCTCGGTCCTGTCTGA-TGA-3'; Reverse 5'-TGGTACCCGTCAGGGTGT-TG-3')

β-Actin (Forward, 5'-AGCCATGTACGTAGCCAT-CC-3'; Reverse, 5'-TTCACAGGTGGGATTGAAG-AG-3')

2.7. Enzyme-Linked Immunosorbent Assay (ELISA). BV2 cells (2 × 10^5 cells/well in 96 well plates) were treated with LPS in the presence or absence of TCA (10 µM) for 6h. To determine the amount of IL-1β and TNF-α secreted, the supernatants of the cells were collected and analyzed using commercially available enzyme-linked immunosorbent assay kits purchased from Invitrogen according to manufacturer's protocol. Experiments were repeated at least three times.

2.8. Transfection and Dual Luciferase-Reporter Assay. For measurement of NF-κB transcriptional activity, BV-2 cells were seeded at a density of 5 × 10^4 cells/well in a 24-well plate. The triplicate wells were cotransfected with a mixture of plasmid (pNF-κB-luc reporter plasmid and PRL-TK internal Control plasmid) using lipofectamine transfection reagent according to manufacturer's specifications (Promega, Madison, WI, US). After 24h transfection, the cells were pretreated with TCA (10 µM) for 2h followed by LPS (0.1 µg/ml) stimulation for 6h. The cells were harvested in lysis buffer and were analyzed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega). The firefly luminescence was quantified, standardized to Renilla expression and reported as relative activity.

2.9. Immunofluorescence Staining. BV2 cells were treated with LPS in the presence or absence of TCA (10 µM) for 24h in 24-well plate followed by three times with PBS. The medium was removed and cells were fixed with ice cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 15min. Cells were permeabilized with 0.2% Triton X-100 for 10min followed by blocking with 10% normal donkey serum (Jackson ImmunoResearch Lab, West Grove, PA, USA) for 30min at room temperature. Fixed cells were incubated with anti-CD11b monoclonal antibody (1:500) in PBS containing 0.1% Triton X-100 overnight at 4℃ and then Alexa Fluor 594-conjugated donkey anti-mouse secondary antibody (1:500; Invitrogen) for 1h at room temperature. The nuclei were counterstained using DAPI. Images of microglia were visualized under an Axiovert 40 CFT visible/fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

3.1. Effects of TCA on NO Production and Cytotoxicity in LPS-Stimulated BV2 Cells. Since NO production is an excellent indicator of inflammatory response, we evaluate the effects of TCA on LPS-induced NO production in BV2 cell lines. Griess assay showed that TCA dose-dependently diminished LPS-induced NO production in BV2 cells (Figure 1(a)). In a parallel experiment, we compared the effectiveness of TCA to block LPS-induced NO production with the well-known microglial deactivator minocycline (Mino) [28, 29]. TCA at dose of 10 µM displayed similar level of inhibition seen with 50 µM minocycline in BV2 lines (Figure 1(b)). Moreover, to determine the cytokotoxic effects of TCA and minocycline on BV2 cells, cells were treated with varying concentrations of TCA and minocycline for 24h. MTT assay showed that TCA up to 10 µM and minocycline 50 µM displayed no significant effects on cell viability in BV2 cells (Figures 1(c) and 1(d)). Since TCA is not toxic to BV2 lines up to 10 µM, these results suggest that TCA at the concentration of 2.5–10 µM can be safely used to block LPS-induced inflammatory responses in BV2 cells.

3.2. Effects of TCA on the Protein Levels of Proinflammatory Mediators in LPS-Stimulated BV2 Cells. To clarify molecular mechanism associated with the suppressive effects of TCA on LPS-induced production of proinflammatory mediators,
we analyzed the protein levels of iNOS, COX-2, IL-1β, and TNF-α in BV2 cells stimulated by LPS. Western blot analysis showed that significant upregulation of iNOS and COX-2 expression could be detected in BV2 cells upon LPS stimulation for 24 h, while pretreatment of TCA at dose of 2.5 μM and 5 μM did not significantly prevent LPS-induced upregulation of iNOS and COX-2 expression. Instead, TCA at dose of 10 μM led to a dramatic reduction of iNOS and COX-2 expression at 24 h after LPS stimulation compared to LPS-stimulated microglia without TCA pretreatment (Figures 2(a) and 2(b)). Similar effect was observed with the levels of IL-1β release judged by ELISA analysis. ELISA showed that LPS stimulation for 6 h more than doubled the levels of TNF-α and IL-1β release in BV2 cells, while pretreatment of TCA at 10 μM almost completely abolished LPS-induced IL-1β release (Figure 2(c)). TCA pretreatment did not affect the secretion of TNF-α in LPS-stimulated BV2 cells (Figure 2(d)).

3.3. Effects of TCA on the mRNA Expression of Proinflammatory Mediators in LPS-Stimulated BV2 Cells. Because of the importance of proinflammatory mediators in chronic inflammation, we examined the effects of TCA on LPS-induced mRNA expression of proinflammatory factors, including iNOS, COX-2, IL-1β, and TNF-α, in BV2 cells by qRT-PCR analysis. The results showed that LPS stimulation significantly increased the mRNA expression of iNOS, COX-2, IL-1β, and TNF-α at varying times (Figure 3), while pretreatment of TCA blocked LPS-induced iNOS and COX-2 mRNA at a later time point (after 24 h, Figures 3(a) and 3(b)) and suppressed the upregulation of IL-1β mRNA at early time point (before 6 h, Figure 3(c)) but displayed no effects on TNF-α mRNA in LPS-stimulated BV2 cells (Figure 3(d)).

3.4. TCA Recovers LPS-Induced Morphological Alteration in BV2 Cells. Since the morphological alteration of microglia can be observed upon inflammatory condition, we treated BV2 cells with LPS (0.1 μg/ml) in the absence or presence of TCA (10 μM) for 24 h. Anti-CD11b immunofluorescence staining analysis showed that resting BV2 cells exhibited elongated cell bodies and retracted processes, indicating a morphological transformation into amoeboid/activated. However, TCA pretreatment partially blocked LPS-induced morphological
alteration and microglial activation, and short ramified processes were observed in BV2 cells (Figure 4).

3.5. Inhibitory Effects of TCA on NF-κB Activation and IκBα Phosphorylation in LPS-Stimulated BV2 Cells. The activation of NF-κB by LPS stimulation can induce the expression of proinflammatory mediators, which contribute to the pathogenesis of the inflammatory process [27]. We investigated the regulation of NF-κB activation by TCA using Western blot of nuclear NF-κB analysis and Dual Luciferase-reporter assay. The transfected BV2 cells were pretreated with TCA (10 μM) for 2 h before the LPS (0.1 μg/ml) stimulation for 6 h. We examined the effects of TCA on the nuclear translocation of the p65 subunit of NF-κB in LPS-stimulated BV2 cells. The NF-κB activation was induced by LPS stimulation. It also caused the nuclear translocation of the p65 (cytosol), subunit of NF-κB. The above process was dramatically suppressed by TCA (Figure 5(a)). Further, the cells were cotransfected with pNF-κB-luc reporter and PRL-TK plasmid. After transfection, cells were treated with TCA (10 μM) for 2 h before the 6 h LPS stimulation (0.1 μg/ml), and then NF-κB transcriptional activity was determined and expressed as relative luciferase activity (RLU). As shown in Figure 5(b), NF-κB transcriptional activity was significantly enhanced by LPS stimulation, while the enhancement of NF-κB activity was inhibited by TCA pretreatment. In addition, we also investigated the effects of TCA on the cytosolic expression of p-IκBα and IκBα in LPS-stimulated BV2 cells. Figure 5(c) showed that pretreatment of TCA decreased LPS-induced phosphorylation of IκBα, which further indicated

**Figure 2:** Effects of TCA on the protein levels of iNOS, COX-2, IL-1β, and TNF-α in LPS-stimulated BV2 cells. BV2 cells were pretreated with 2.5–10 μM TCA for 2 h prior to 0.1 μg/ml LPS stimulation. After 24 h, cells were lysed and cell lysates were subjected to Western blot to detect iNOS (a) and COX-2 (b) expression. The protein levels of iNOS and COX-2 were standardized based on the respective level of β-actin protein. Value was expressed as relative changes in comparison to Control, which was set to 1. Supernatants were collected at 6 h after LPS stimulation and subjected to ELISA to measure the amount of IL-1β (c) and TNF-α (d) secreted by BV2 cells with TCA 10 μM pretreatment. Each value indicates the mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 versus untreated Controls; #P < 0.05, ##P < 0.01, and ###P < 0.001 versus LPS alone.
that the subsequent NF-κB inactivation was induced by TCA in LPS-stimulated BV2 cells. Together, these results suggested that the inhibition of NF-κB activation by TCA may be the mechanism responsible for the suppression of proinflammatory mediators in LPS-stimulated BV2 cells.

3.6. Attenuation of Microglial Neurotoxicity in the Microglia/Neuron Coculture by TCA Pretreatment. Excessively activated microglia through the release of various proinflammatory mediators are well recognized as a major contributing factor to neuronal degeneration [30]. We thus carried out microglia/neuron coculture system to investigate the potential neuroprotective effects of TCA on microglial neurotoxicity (Figure 6(a)). We plated BV2 cells in the inserts and PC12 cells in the underwells, which have been shown to allow the free exchange of proinflammatory cytokines without direct contact between BV2 and PC12 cells [31, 32]. The coculture experiments revealed that LPS-stimulated BV2 cells decreased the viability of PC12 cells while unstimulated BV2 cells did not. However, pretreatment of PC12 cells with TCA significantly improved cell viability (Figure 6(b)). These results implicate that TCA may possess the capability to protect neuronal cells from being damaged by suppressing activated microglia-mediated inflammation.

3.7. The Effects of TCA and JSH-23 on NO Production and Neurotoxicity in LPS-Stimulated BV2 Cells and Microglia/Neuron Coculture. As NF-κB signaling pathway may be involved in the inhibitory effects of TCA on proinflammatory mediators in LPS-stimulated BV2 cells, we subsequently investigated the effects of JSH-23, an inhibitor of NF-κB nuclear translocation, on NO production in LPS-stimulated BV2 cells and neurotoxicity in microglia/neuron coculture. Griess assay showed that JSH-23 decreased the level of NO production
Figure 4: Effects of TCA on LPS-induced morphological alteration in BV2 cells. Immunostaining of BV2 cells was performed by using anti-CD11b monoclonal antibody (red) in the presence of DAPI (blue). Staining was visualized under a fluorescence microscopy. Magnification is 20x and scale bar is 100 μm. Insets show high-magnification representative BV2 cells. Control: untreated BV2 cells. Treatment condition: TCA: TCA (10 μM) treatment alone. LPS: LPS (0.1 μg/ml) stimulation alone. LPS + TCA: combined treatment of LPS (0.1 μg/ml) and TCA (10 μM).

4. Discussion

Under the neurodegenerative condition, microglia can be activated and release a variety of neurotoxic and proinflammatory mediators such as iNOS, COX-2, IL-1β, and TNF-α which are associated with severe neuronal damage and progression of neuroinflammation [9, 33]. Neuroinflammation is a host defense mechanism for protecting the central nervous system (CNS) against aging, infection, and injury. However, sustained neuroinflammatory responses can contribute to neuronal damage and memory impairment in neurodegenerative disorders. Therefore, the suppression of microglial activation and subsequent neuroinflammation have been considered as an effective therapeutic strategy to alleviate the progression of neuroinflammation-mediated neurodegenerative disorders.

Both in vitro and in vivo studies have demonstrated anti-inflammatory potential of TCA, the major constituent from C. cassia [21, 34]. In this study, we investigated the possibility of using TCA to prevent neuroinflammation-caused neuronal damage. Our results showed that TCA was capable
Figure 5: Inhibitory effects of TCA on NF-κB activation and IkBα phosphorylation in LPS-stimulated BV2 cells. (a) Total nuclear protein was extracted to detect NF-κB expression followed by Western blot using an anti-NF-κBp65 antibody. Quantification of protein band densities was normalized to the corresponding levels of Histone 3. (b) Effects of TCA on LPS-induced NF-κB luciferase activity in BV2 cells. (c) Total cytosolic protein was extracted to detect p-IκBα expression, which were normalized with the levels of total IκBα. Value was expressed as relative changes in comparison to Controls, which was set to 1. Data are the mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 versus untreated Controls; #P < 0.05, ##P < 0.01, and ###P < 0.001 versus LPS alone.

Furthermore, we determined the effects of TCA (10 μM) on the mRNA expression of iNOS and COX-2 in LPS-stimulated BV2 cells for varying times (Figure 3). We found that pretreatment of TCA blocked LPS-induced iNOS and COX-2 mRNA at later time point (after 24 h, Figures 3(a) and 3(b)). The same inhibitory effects of TCA were observed on IL-1β mRNA and production in LPS-stimulated BV2 cells (Figures 2(c) and 3(c)), but TCA displayed no effects on TNF-α mRNA and production in LPS-stimulated microglia (Figures 2(d) and 3(d)). Subsequently, we observed that TCA also prevented the conversion of resting microglia into activated ones in LPS-stimulated BV2 cells (Figure 4). The suppressive effects of TCA on microglial activation by
Ronal death is the excessive production of proinflammatory microglial activation-induced neuroinflammation and neuronal damage [27]. The most likely mechanism behind microglial activation-induced neuroinflammation and neuronal death is the excessive production of proinflammatory mediators that are neurotoxic [39–41]. For example, iNOS expression and NO production are upregulated in activated microglia [42, 43]. Anti-inflammatory agents have been shown to inhibit microglial activation and production of proinflammatory mediators. Importantly, these agents are able to attenuate neuronal degeneration and thus exert neuroprotective effects [44, 45]. The fact that TCA is able to reduce proinflammatory mediators expression in LPS-stimulated BV2 cells suggests that TCA may grant protective effects to neuronal cells under inflammation. In this study, our data showed that LPS-stimulated BV2 cells conferred significant toxicity to PC12 neuronal cells in a transwell-based coculture system. However, pretreatment of TCA significantly improved PC12 cell survival (Figure 6). To determine whether the NF-κB signaling pathway is involved in the neuroprotective effects of TCA in LPS-stimulated microglia/neuron coculture, we treated LPS-stimulated BV2 cells with specific inhibitor, JSH-23, to block NF-κB signaling pathway followed by improving PC12 cells survival. Since combined treatment of JSH-23 and TCA had similar effects on NO production and neuronal survival as JSH-23 used alone, we conclude that TCA inhibits microglial neurotoxicity by interfering with NF-κB signaling pathway (Figure 7(b)). This observation raises the possibility of using TCA as a natural product to deter neurodegenerative process.

5. Conclusions
As illustrated in the summary diagram (Figure 8), we provide the evidences that natural product TCA can protect neuronal damage under neuroinflammatory condition and TCA accomplishes its role by suppressing microglial activation and proinflammatory mediators expression via blocking NF-κB signaling pathway in LPS-stimulated BV2 cells. Our study suggests that TCA, a natural product, may be

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**Figure 6: TCA attenuated microglial neurotoxicity in the microglia/neuron coculture.** BV2 cells were cocultured with PC12 cell using transwell system. (a) The experimental timeline for microglia/neuron coculture. BV2 cells were pretreated with or without TCA (10 μM) for 2 h and then stimulated with LPS (0.1 μg/ml) prior to coculture. (b) After 24 h of coculture period, transwell inserts were removed and MTT assay was performed to determine the viability of PC12 cells in the underwells. Data are the mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 versus untreated Controls; #P < 0.05, ##P < 0.01, and ###P < 0.001 versus LPS alone.
Figure 7: TCA inhibited LPS-induced NO production in BV2 cells and neurotoxicity in microglia/neuron coculture by intercepting the NF-κB signaling pathway. (a) BV2 cells were stimulated with LPS (0.1 μg/ml) in the absence or presence of TCA (10 μM), JSH-23 (25 μM), or TCA + JSH-23 for 24 h followed by Griess assay to measure the level of nitrite in medium. (b) MTT assay was performed to analyze PC12 cells viability in microglia/neuron coculture with LPS stimulation in the absence or presence of TCA (10 μM), JSH-23 (25 μM), or TCA + JSH-23 for 24 h. Data are the mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 versus untreated Controls; #P < 0.05, ##P < 0.01, and ###P < 0.001 versus LPS alone.

Figure 8: Schematic diagram representing TCA’s inhibition of microglial activation and improvement of neuronal survival by blockage of NF-κB activation.

represented as a potential therapeutic agent for ameliorating neuroinflammation-mediated neurodegenerative diseases.

Additional Points

Highlights. TCA suppresses microglial activation and proinflammatory mediators expression via blocking NF-κB signaling pathway in LPS-stimulated BV2 cells. TCA can protect neuronal damage under neuroinflammatory condition. TCA is a potential therapeutic natural product for ameliorating neuroinflammation-mediated neurodegenerative diseases.

Disclosure

This manuscript is an extended version of the conference abstract that has been presented in “Chinese Society of Physiology 24th National Congress and Physiology Conference Proceedings,” 2014 (conference abstract link: http://cpfd.cnki.com.cn/Article/CPFDTOTAL-OGSC201410001282.htm).

Conflicts of Interest

The authors declare no financial conflicts of interest.

Authors’ Contributions

Ying Xu and Yongjun Chen conceived and designed the research. Yan Fu, Pin Yang, Yang Zhao, Xianwen Dong, and Zhongping Wu performed the research. Liqing Zhang and Zhangang Zhang conducted preliminary experiment. Ying Xu and Yongjun Chen analyzed the data and prepared the manuscript. All authors read and approved the final manuscript. Yan Fu, Pin Yang, and Yang Zhao contributed equally to this work.
Acknowledgments

This work was supported by grants from General Program of National Natural Science Foundation of China (Grants 81274119 and 81773927 to Ying Xu and Grant 31571041 to Yongjun Chen) and Innovation Program of Shanghai Municipal Education Commission (Grant 13YZ050 to Ying Xu).

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