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Neuroprotective role of tripchlorolide on inflammatory neurotoxicity induced by lipopolysaccharide-activated microglia

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ABSTRACT

A large body of evidence has suggested a strong association between neuroinflammation and the pathogenesis of many neurodegenerative diseases. Therefore, it is a good target for therapeutic treatment. So far, studies have proven anti-inflammatory herbal medicine and its constituents to be effective in slowing down the neurodegenerative process. The present study tested tripchlorolide, an extract of *Tripterygium wilfordii* Hook F (TWHF), as a novel agent to suppress inflammatory process in microglia. It showed this novel agent to be cytotoxic at a dose of 20–40 nM to primary microglia and BV-2 microglial cells but not to primary cortical neurons and Neuro-2A cells *in vitro*. Moreover, tripchlorolide protected primary cortical neurons and Neuro-2A cells from neuroinflammatory toxicity induced by the conditioned media from lipopolysaccharide (LPS)-stimulated microglia, which resulted in a significant decrease in their cell survival. The changes of the inflammatory mediators in this process were further investigated. In the LPS-stimulated microglia, the production of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), nitric oxide (NO), prostaglandin E_2 (PGE_2), and intracellular superoxide anion (SOA) was markedly attenuated by tripchlorolide at a dose of 1.25–10 nM in a dose-dependent manner. Furthermore, the production of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) was also significantly inhibited by tripchlorolide in both mRNA and protein levels. These results suggest that tripchlorolide can protect neuronal cells via a mechanism involving inhibition of inflammatory responses of microglia to pathological stimulations. Therefore, it is potentially a highly effective therapeutic agent in treating neuroninflammatory diseases.

1. Introduction

The pivotal role of inflammation-mediated mechanisms in the neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) has evolved from a hypothesis to mainstream thinking. The hallmark of brain inflammation is the activation of microglia [1,2]. In pathological situations, activated microglia cells experience dramatic morphological changes from resting ramified cells to motile activated amoeboid microglia. Once immuno-stimulated in response

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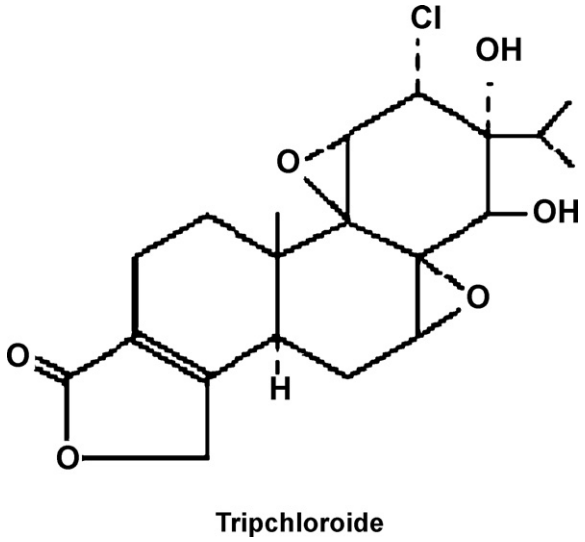

Fig. 1 – The chemical structure of tripchlorolide (T4).

to neuro-degenerative events, these cells release a variety of proinflammatory mediators, such as cytokines, reactive oxygen species, complement factors, free radical species, and nitric oxide (NO), which can all contribute to both neuronal dysfunction and cell death, ultimately creating a vicious cycle [3,4].

Based on these theories, a number of clinical trials with anti-inflammatory agents have been conducted on neuro-degenerative disease patients, such as AD [5,6]. Extracts of Chinese herb *Tripterygium wilfordii* Hook F (TWHF) have been found to have potent anti-inflammatory and immunosuppressive functions and are widely used in China for treatment of rheumatoid arthritis [7,8]. One of the major active ingredients of TWHF is triptolide (PG490), a diterpene triepoxide. Treatment of rat with triptolide (5 μg/kg, intraperitonelly, once a day for 24 days) dramatically improved the survival rate of DA neurons by inhibiting microglial activation and the releases of proinflammatory cytokines in lipopolysaccharide (LPS) intranigrally lesion rat model [9]. Recently, it has also been demonstrated to upregulate both NGF mRNA and protein level in rat astrocyte culture at a dose of 10 nmol/L [10]. Tripchlorolide (designated as T4, Fig. 1), a novel analog of triptolide, has immunosuppressive activities similar to those of triptolide but with greatly reduced toxicities, such as gastrointestinal action [11]. More importantly, its lipophilic character generated by chloridion modification, together with its small molecular size (MW 397), probably facilitates it passage through the blood–brain barrier. In fact, Li et al. firstly reported that *in vivo* administration of tripchlorolide (1 μg/kg, intraperitonelly, for 28 days) increased the survival of dopaminergic (DA) neurons in substantia nigra pars compata (SNpc) by 67% in a rat model of transaction of medial forebrain bundle [12]. It also significantly improved the level of dopamine in the substantia nigra and striatum to 157 and 191%, respectively, *in vivo* in the PD model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned C57BL/6 mice [13]. Taken together, these studies strongly suggest that tripchlorolide may be a potential neurotrophic and neuroprotective agent against inflammatory challenges in central nervous system (CNS).

LPS-activated microglial cells that mimic inflammation have been proven to be a good cellular model for screen potential therapeutic compounds for neuroinflammatory disorders [14,15]. This study was therefore designed to evaluate the effects of tripchlorolide on the cell survival of primary cortical neurons, Neuro-2A neuronal-like cells, primary microglia and BV-2 microglial cells and to investigate its effects on the survival of primary cortical neuron and Neuro-2A cell in conditioned media from LPS-stimulated microglia. In order to gain insights into the molecular mechanisms, this study also examined the levels of several inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), NO, prostaglandin E_2 (PGE_2), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), as well as intracellular superoxide anion (SOA) in microglia.

2. Materials and methods

2.1. Materials

T4 was obtained from the Department of Pharmacology of Fudan University (Shanghai, China). The material was in the form of white needle-like crystals, with a melting point of 256–258 °C, molecular weight 397, and 98% purity by reverse phase high-pressure liquid chromatography (HPLC) evaluation.

Dulbecco's modified Eagel's medium (DMEM), DMEM-F12, Hanks' balanced salt solution (HBSS), Neurobasal media with L-glutamine, B27 supplements and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Trizol reagent was purchased from Invitrogen (Carlsbad, CA). LPS (from *Escherichia coli* serotype O111: B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), *tert*-butyl hydroperoxide (t-BHP), N-acetyl-L-cysteine (NAC) and nitroblue tetrazolium (NBT) were obtained from Sigma (St. Louis, MO). Primary antibodies, including rabbit ploclonal antibodies to mouse iNOS, COX-2, MAP-2, were purchased from Cayman (Ann Arbor, MI), Cell signaling (Berverly, MA) and Chemicon, Inc. (Temecula, CA), respectively. 4′,6-Diamidine-2′-phenylindole, dihydrochloride (DAPI), FITC-conjugated second antibody and Western blot Chemiluminescent Detection System (LumiGLO system) were from KPL (Gaithersburg, MD). TNF-α, IL-1β ELISA kits were from BioSource (Camarillo, CA) and PGE_2 EIA kit from Cayman (Ann Arbor, MI). Other regents were analytical or cultured grade purity.

2.2. Cell culture and treatment

2.2.1. BV-2 and Neuro-2A cells culture

Murine BV-2 microglial cells and Neuro-2A neuronal-like cells were maintained in Dulbucco's modified Eagle medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin, and kept at 37 °C in humidified 5% CO_2/95% air. The cells were passaged every 3 days when growing up to 75% confluence.

2.2.2. Primary cortical neurons culture

Neurons were cultured from cortices of embryonic day 16 (E16) C57BL/6 mice. Briefly, meninges-free cortices were isolated, indigested with 0.05% trypsin–EDTA, and plated onto poly-L-lysine (0.05 mg/ml)-coated tissue culture wells (1000 cells/mm^2) for 6 days *in vitro* before use. Neurons were grown in Neurobasal media with L-glutamine and B27 supplements to consistently provide neuronal cultures to 95% purity and assessed by staining with antibody directed against the neuronal marker anti-microtubule-associated protein 2 (MAP-2).

2.2.3. Primary microglia culture

Microglia were derived from postnatal day 1 (P1) mouse brains (C57BL/6). Briefly, meninges-free cortices from postnatal day 1 mice were isolated and trypsinized. Cells were plated onto tissue culture plastic in DMEM-F12 with L-glutamine containing 10% heat-inactivated FBS and fed every 3 days. After 14 days, the cultures were shaken vigorously (120 min; 260 rpm on a rotary shaker) to remove microglia [16].

2.2.4. LPS, tripchlorolide and conditioned media treatment

T4 was first dissolved in dimethyl sulfoxide (DMSO) and later mixed with serum free culture medium to give an appropriate concentration with a maximum of 0.01‰ DMSO. LPS was diluted in Hanks' balanced salt solution (HBSS) to a concentration of 2 mg/ml, aliquoted and stored at −20 °C until use.

For inflammatory mediators assay, primary microglia and BV-2 cells were stimulated with 1 μg/ml LPS for indicated time [17]. For tripchlorolide treatment, primary microglia and BV-2 cells were treated with various concentrations of tripchlorolide 1 h prior to stimulation with LPS. Tripchlorolide was not rinsed away in this process.

For the conditioned media experiments, Neuro-2A cells were plated at a density of 2×10^4 cells per well in 96 well plates and allowed to settle for 24 h before replacement with conditioned media. Primary microglia and BV-2 cells were treated respectively with tripchlorolide (1.25–10 nM) for 1 h prior to 1 μg/ml LPS stimulation (T4 + LPS-CM group), respectively with LPS alone (LPS-CM), and respectively with HBSS (vehicle), each treatment lasting for 24 h. Then the BV-2 microglial and primary microglia-conditioned media, collected and clarified by centrifugation, were transferred respectively to Neuro-2A cells and primary cortical neurons for a further 48 h. The groups of HBSS-induced conditioned media were set as control. The survival of Neuro-2A cells and primary cortical neurons treated with LPS (0.1–10 μg/ml) alone for 48 h was also observed.

2.3. Cell viability assay

2.3.1. MTT assay

MTT was converted in living cells to formazan, which has a specific absorption maximum. Cells were treated with LPS and/or tripchlorolide for indicated times, and further incubated for 4 h when the culture medium was changed to the medium containing 0.5 mg/ml MTT. Then, they were added with solubilization solution (10% SDS, 5% isopropanol in 0.012 M HCl) and incubated at 37 °C in humidified 5% CO_2/95% air for overnight. The absorbance of the supernatant was measured at 570 nm on an automated microtiter plate reader. Data were expressed as the mean percentage of viable cell versus control.

2.3.2. Immunocytochemistry

Quantification of neuron survival was assessed by immunocytochemistry. To perform culture immunocytochemistry, neurons were stimulated as described above and fixed in 4% paraformaldehyde at 37 °C for 30 min at select times. To assess survival, they were stained with an anti-MAP2 antibody. Antibody binding was visualized by using FITC-conjugated secondary antibody. Cell nuclei were counterstained with DAPI. Viable neurons were then enumerated under a fluorescence microscope.

2.4. TNF-α, IL-1β and PGE_2 measurement

Primary microglia and BV-2 cells were seeded on 24-well plates. After being treated with LPS and/or tripchlorolide, the supernatants were collected and stored at −80 °C until assays for TNF-α, IL-1β and PGE_2 were performed. TNF-α, IL-1β and PGE_2 levels were detected by mouse TNF-α, IL-1β ELISA kits and PGE_2 EIA kit according to the procedures provided by the manufacturers.

2.5. Nitrite quantification

NO_2^- accumulation in the medium was used as an indicator of NO production as previously described [18].The isolated supernatants were mixed with an equal volume of Greiss reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 15 min. $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring optical density at 540 nm.

2.6. Modified NBT assay for SOA

A modified assay for the intracellular conversion of nitroblue tetrazolium (NBT) to formazan by superoxide anion (O_2^-) was used to measure the production of reactive oxygen species [19,20]. In brief, 0.1% NBT was added to the media at the end of the treatment periods. As negative controls, BV-2 cells were pretreated with 10 mM NAC 1 h prior to LPS treatment. As a positive control, BV-2 cells were treated with 100 μM t-BHP for 60 min [21,22]. After incubation for 45 min at 37 °C, the cells were washed twice with warm PBS, then once with methanol, and air-dried. The NBT deposited inside the cells was then dissolved with 240 μl of 2 M potassium hydroxide (KOH) and 280 μl of dimethylsulfoxide (DMSO) with gentle shaking for 10 min at room temperature. The dissolved NBT solution was then transferred to a 96 well plate and absorbance was read on a microplate reader at 630 nm. Meanwhile, the cells (1×10^5/well) were allowed to adhere to glass cover slips placed in a 6-well flat culture plate. After similar treatments, NBT incubation, washing and fixing with methanol were carried out. The cells containing blue formazan particles (NBT-positive cells) were pictured under a microscope.

2.7. *Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis*

A semiquantitative RT-PCR assay was used to determine the mRNA levels of iNOS, COX-2 in relation to β-actin. Total RNA was extracted by Trizol reagent. The RT-PCR assays were performed with a Titan™ One Tube RT-PCR System kit (Boehringer Mannheim, Germany). Briefly, 0.5 μg of total RNA from each sample was added to 25 μl of a reaction mixture. The primer sequences were as follows: sense 5′-CTG CAG CAC TTG GAT CAG GAA CCT G-3′ and antisense 5′-GGG AGT AGC CTG TGT GCA CCT GGA A-3′ for iNOS (gene bank number: BC062378.1, 310 bp fragment); sense 5′-TTG AAG ACC AGG AGT ACA GC-3′ and antisense 5′-GGT ACA GTT CCA TGA CAT CG-3′ for COX-2 (gene bank number: BC052900.1, 324 bp fragment); sense 5′-GGC ATG GGT CAG AAG GAT TCC-3′ and antisense 5′-ATG TCA CGC ACG ATT TCC CGC-3′ for β-actin (gene bank number: BC014861.1, 500 bp fragment). Then these three genes were amplified using different-temperature PCR system consisting of denaturation at 94 °C for 45 s, primer annealing at 60 °C (for iNOS) or 58 °C (for COX-2) for 45 s, and extension at 70 °C for 1 min. The number of cycles was determined for samples not reaching the amplification plateau (27 cycles for iNOS and 25 cycles for COX-2). The amplified conditions for β-actin were the same to those for iNOS or COX-2. The PCR product was visualized by electrophoresis in a 1.6% agarose gel. The verification of specific genes was established by their predicted size under UV light. Quantification of the band density was performed by densitometric analysis (Digital Image Analysis System, PDI, Alpha, USA), and calculated as the optical density × area of band. The mRNA of iNOS and COX-2 was evaluated by integrated density value (IDV) as $IDV_{iNOS/\beta\text{-actin}}$ and $IDV_{COX\text{-}2/\beta\text{-actin}}$.

2.8. *Western blot analysis of iNOS and COX-2 proteins*

The treated cells were washed with ice-cold PBS and then were incubated with lysis buffer containing 10 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 0.1% SDS, 0.5% (w/v) sodium deoxycholate, 1% Triton-X 100, 1 mM PMSF, 60 μg/ml aprotinin, 10 μg/ml leupeptin and 1 μg/ml pepstatin for 20 min. Then the cells lysates were centrifuged at 12,000 × *g* for 10 min, and the protein concentrations were determined by the Bradford method. Then 40 μg of total cell protein was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were incubated with the appropriate antibody (iNOS and β-actin for 1:1000, COX-2 for 1:750) using Western blot chemiluminescence detection system. Quantification of the band density was performed by densitometric analysis.

2.9. *Statistical analysis*

Data were analyzed by SigmaStat 3.5 software and shown by the means ± S.D. of at least three independent experiments. Statistical differences between values were determined by Student's *t*-test (when data were normalized as %control) or ANOVA followed by *Tukey post hoc* test (other data). Significance level was set at $P < 0.05$.

3. Results

3.1. *Effects of tripchlorolide on the survival of primary cortical neurons, primary microglia, Neuro-2A and BV-2 cells*

We investigated the effects of tripchlorolide on the survival of neuronal and glial cells, including primary cortical neurons, Neuro-2A, primary microglia and BV-2 cells by MTT reduction assay. All the cells were treated with a variety of tripchlorolide (5–80 nM) for 48 h. As shown in Fig. 2, tripchlorolide at range of 20–80 nM markedly decreased the percentage of survival in primary microglia and BV-2 cells. The IC50 of them was 75.84 and 29.74 nM, respectively. However, in primary cortical neurons treated with tripchlorolide (high at 80 nM) there were no significant changes on cell survival, and in Neuro-2A cells, a small decrease (20%) when T4 was at a dose of 80 nM. These data indicate that tripchlorolide in the range of 20–80 nM is cytotoxic to microglial cells, but not to primary cortical neurons.

3.2. *Effects of tripchlorolide on cell survival of neuronal cells treated with conditioned media from LPS-stimulated microglia*

The present study firstly confirmed that the conditioned media from LPS-stimulated microglia (LPS-CM) were potently toxic to Neuro-2A cells and primary cortical neurons. As shown in Fig. 3, LPS-CM resulted in approximately 57% reduction in the viability of the former (Fig. 3B column 2) and approximately 52% reduction in the latter (Fig. 3C column 5) ($P < 0.001$). There were no significant changes in the survival ratio of Neuro-2A cells and primary cortical neurons when

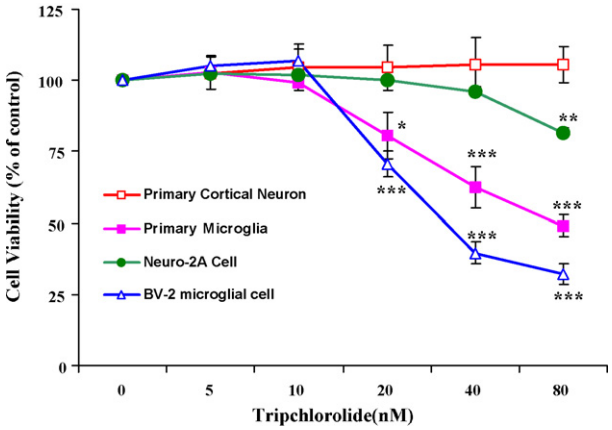

Fig. 2 – Effects of tripchlorolide (T4) on the cell viability. Primary fetal mouse cortical neurons, primary mouse microglia, Neuro-2A cells and BV-2 cells were treated with T4 (0–80 nM) for 48 h. The cell viability was assessed by MTT reduction assay. The values are expressed as mean ± S.D. (n = 5/group) of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$ compared with control group.

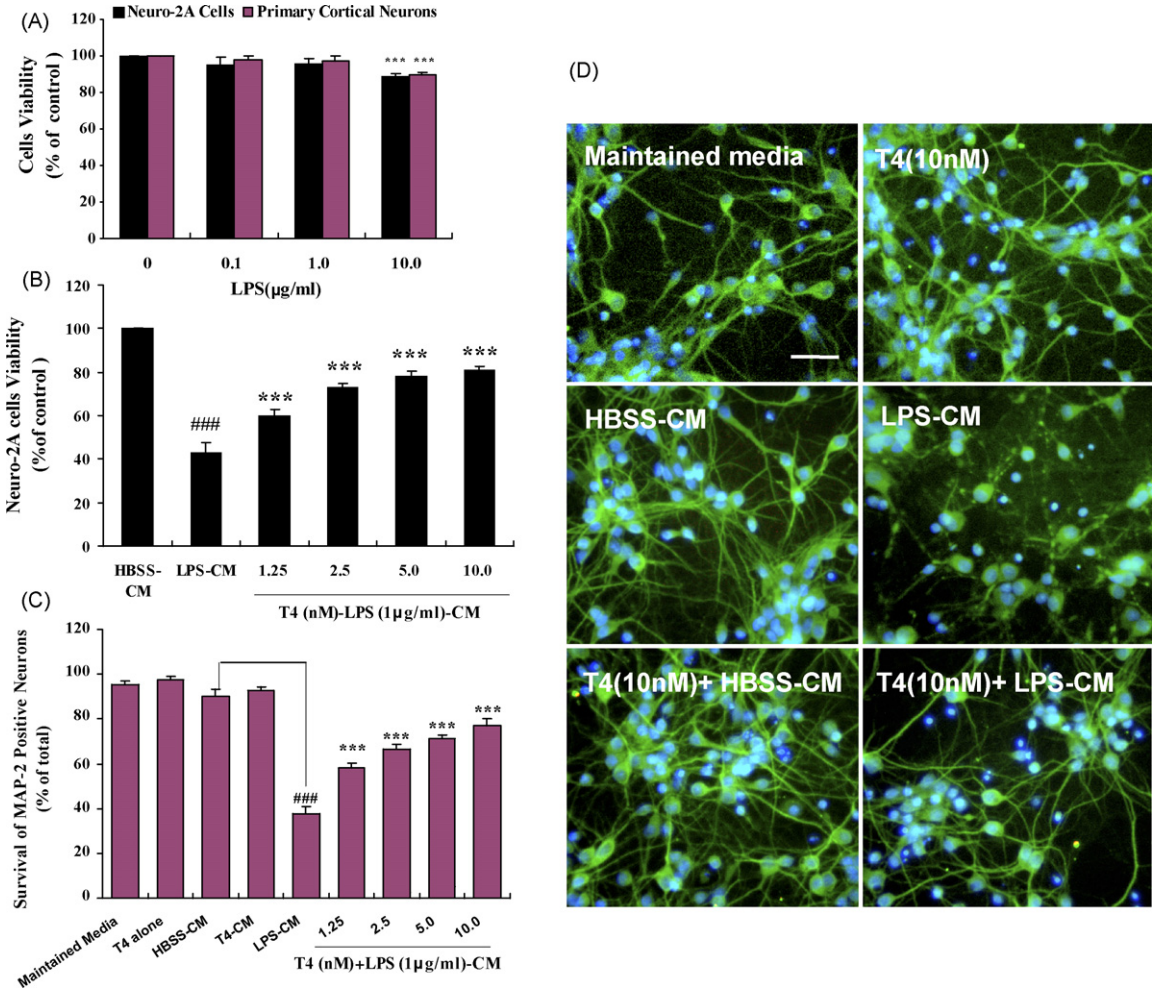

Fig. 3 – Effects of tripchlorolide (T4) on the survival of neuronal cells treated with conditioned media from LPS-stimulated microglia. (A) Media from plates containing increasing concentrations of LPS (0.1, 1.0, 10 μg/ml) alone were transferred to cortical neurons (E16; 6 days *in vitro*) or Neuro-2A cells for 48 h. Conditioned media (CM) from BV-2 cells or primary microglia stimulated for 24 h with or without LPS (1.0 μg/ml), vehicle (HBSS), and T4 (treated for 1 h prior to LPS stimulation, without being rinsed away) was generated. The conditioned media was then transferred to Neuro-2A cells (B) or primary cortical neuron (C) for a further 48 h, respectively. Neuro-2A cell viability was assessed by MTT reduction assay. (D) Primary cortical neurons were fixed in 4% paraformaldehyde, stained using anti-MAP2 antibody with FITC and cellular nucleus was stained with DAPI, the survival of neuron was counted as above. Scale bar = 40 μm. The values are expressed as mean ± S.D. of at least three independent experiments. $^{###}P < 0.001$, compared with control group (HBSS-CM). $^{**}P < 0.01$, $^{***}P < 0.001$, compared with LPS-CM group.

treated with LPS (0.1–1 μg/ml) alone (Fig. 3A). This suggests that the toxic action of the LPS-stimulated microglial-conditioned media is mostly dependent on microglial secreted products and not on contaminating LPS. As expected, in the experiments, pre-treatment with tripchlorolide of conditioned media (T4 + LPS-CM) provided complete neuroprotection from LPS-stimulated microglial-conditioned media (Fig. 3B and C). The group of T4 (10 nM) + LPS-CM resulted in a 40% and 38% increase in the survival of primary cortical neurons and Neuro-2A cells ($P < 0.001$), respectively, compared with the groups of HBSS-CM. In addition, the neuroprotective action of tripchlorolide groups was in a dose-dependent manner.

Primary cultured cortical neurons that were only treated with LPS-CM showed a marked loss of MAP-2 positive neurons and their dendrites (Fig. 3D middle layer right). The survival ratio of MAP-2 neurons in the LPS-CM group was only 38% (Fig. 3C). In contrast, a significant sparing of the MAP-2 positive neurons was observed in the groups pretreated with tripchlorolide, and the dendritic processes surrounding the cortical neurons were largely preserved (Fig. 3D underlayer right). Tripchlorolide at a dose of 10 nM preserved as many as 77% of MAP-2 positive neurons.

3.3. *Effects of tripchlorolide on LPS-induced TNF-α, IL-1β, NO and PGE_2 levels in primary microglia and BV-2 cells*

According to the rationale that proinflammatory mediators are responsible for the neurons death in microglial-conditioned media dependent manner [23–25], we investigated whether tripchlorolide had anti-inflammatory action in LPS-stimulated

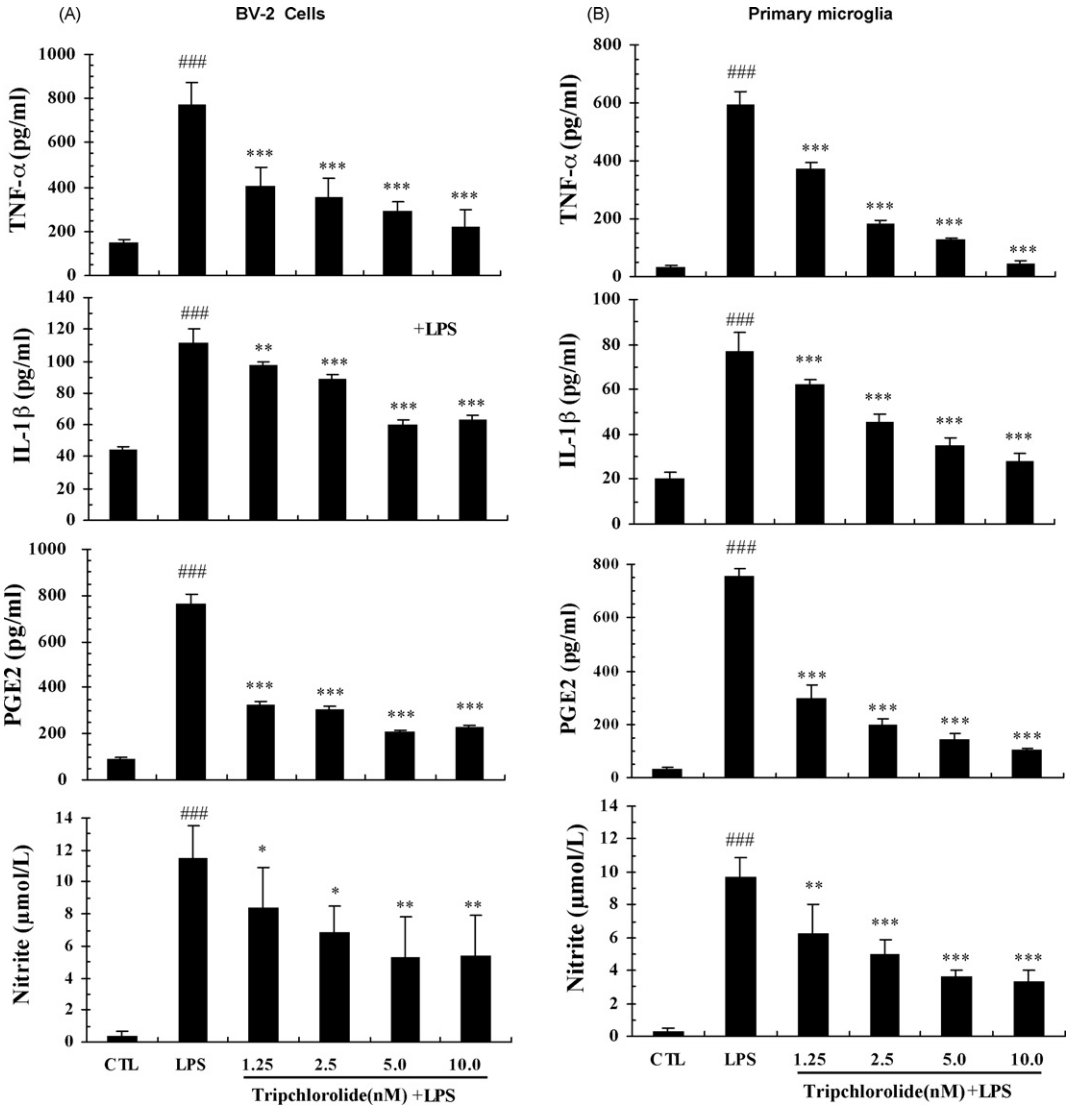

Fig. 4 – Effects of tripchlorolide (T4) on inflammatory mediators in BV-2 cells or primary microglia following LPS exposure. BV-2 cells (A) or primary cultured microglia (B) were pretreated for 1 h with T4 (1.25–10 nM) and then were stimulated with LPS (1.0 μg/ml). After 24 h (TNF-α, NO and PGE_2) or 2 h (IL-1β), an aliquot of the supernatant was collected for ELISA analysis of TNF-α and IL-1β, EIA analysis of PGE_2 and Griess reaction analysis of nitrite levels. Each value is expressed as mean ± S.D. (n = 4/group) of at least three independent experiments. $^{###}P < 0.001$, compared with control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with LPS treated alone.

microglial cells. In an initial time-dependent response study, the exposure of BV-2 cells and primary microglia to LPS produced the maximal response of TNF-α, PGE_2 and NO release at 24 h, as well as IL-1β at 2 h (data not shown). The exposure of BV-2 cells to LPS alone at 1 μg/ml for 24 h resulted in a significant increase in the production of TNF-α, NO and PGE_2 levels to 5.2-, 8.6- and 28.7-fold over basal levels, respectively, as well as IL-1β for 2 h to 2.5-fold (Fig. 4A column 2). However, these proinflammatory mediators were greatly inhibited by tripchlorolide in a dose-dependent manner (Fig. 4A). The pretreatment of cells with tripchlorolide at 5 nM reduced the levels of IL-1β and NO to 46% and 53%, respectively. And tripchlorolide at extremely low dose (1.25 nM) also attenuated the production of TNF-α and PGE_2 to 48% and 57%, respectively. The tripchlorolide-induced decrease in TNF-α, IL-1β, NO and PGE_2 was also observed in the primary microglia cultures (Fig. 4B). This suggests that the anti-inflammatory effect of tripchlorolide is not limited to a particular microglial cell line. It alone did not change the production of cytokines, NO and PGE_2 in BV-2 cells and primary microglia (data not shown).

3.4. *Effect of tripchlorolide on LPS-induced intracellular SOA production in BV-2 cells*

Accumulation of intracellular reactive oxygen species (ROS) triggered the release of inflammatory mediators in microglia [26]. Immortalized microglial cells BV-2 retained the functional characteristics (such as antigen profiles, anti-microbial activities and response to LPS) of primary cultured microglia [27–29]. So, BV-2 cell is a good and convenient cell model to

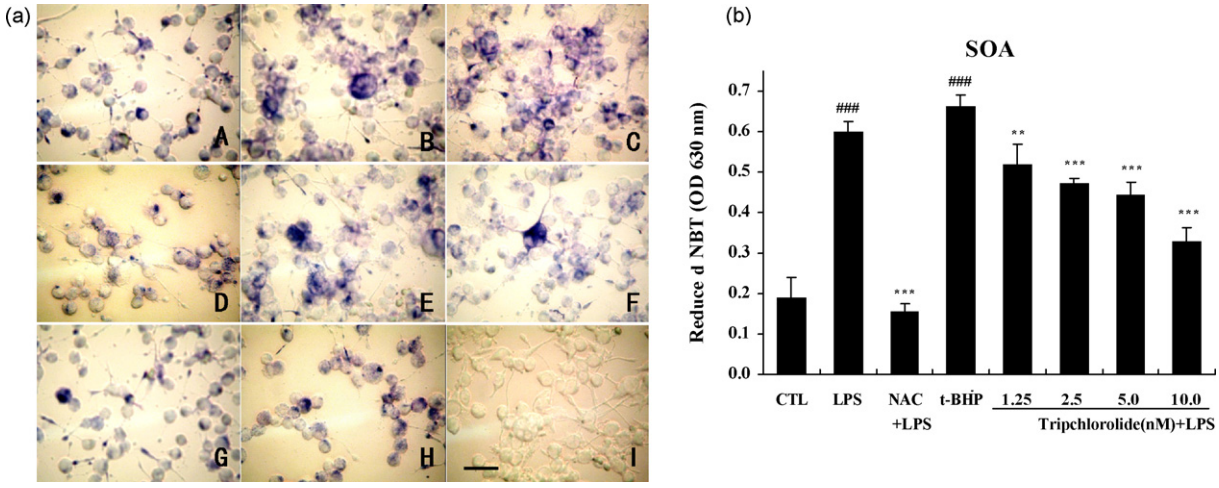

Fig. 5 – Effect of tripchlorolide (T4) on the intracellular level of superoxide anion (SOA) in BV-2 cells following LPS exposure. Nitro blue tetrazolium (NBT) to formazan by superoxide anion (O_2^-) was used to measure the production of SOA. (a) BV-2 cells were pretreated for 1 h with T4 (1.25–10 nM) or 10 mM NAC as negative drug control and then stimulated with LPS (1 μg/ml) for 24 h. Treatment of cells with *t*-BHP (100 μM) alone for 1 h was as a positive model control. Then NBT was incubated for a further 45 min and was fixed with methanol. The cells containing blue formazan particles (NBT-positive cells) were visual under microscope. (A) Drug-free groups; (B) LPS alone; (C) *t*-BHP alone; (D) NAC control; (E–H) pretreated with 1.25, 2.5, 5.0 and 10 nM T4, respectively; (I) blank groups (without NBT). Scale bar = 50 μm. (b) The NBT deposited inside the cells were dissolved with solubilization solution and the superoxide anion levels quantified by the absorbance measured on a microplate reader at 630 nm. Each value is expressed as mean ± S.D. of three independent experiments. $^{###}P < 0.001$, compared with the control group. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with LPS treated alone.

explore the mechanism of microglia-related neuroinflammation in CNS. We therefore investigated whether tripchlorolide could attenuate LPS-induced intracellular ROS production in BV-2 cells. In an initial time dependent response study, the treatment of BV-2 cells with LPS (1 μg/ml) produced the maximal response for SOA generation at 24 h (data not shown). The treatment of BV-2 cells with LPS for 24 h indicated clear NBT positive cell (containing blue formazan particles) (Fig. 5a (B)). The treatment of BV-2 cells with *t*-BHP (100 μM) for 60 min was used as a positive model (Fig. 5a (C)). Interestingly, tripchlorolide treatment markedly inhibited blue formazan particles formation in BV-2 cells (Fig. 5a (E, F, G, H)). NBT reduction quantified assay also revealed the effect of tripchlorolide on SOA in microglia was in a dose-dependent way (Fig. 5b). The maximal inhibitory effect of tripchlorolide (10 nM) was 45%, while *N*-acetyl-L-cysteine (NAC), a free-radical scavenger, resulted in a 74% reduction in the production of SOA induced by LPS (Fig. 5b). Tripchlorolide alone did not change the production of ROA in BV-2 cells (data not shown)

3.5. *Effects of tripchlorolide on the expressions of iNOS, COX-2 mRNA and protein in LPS-stimulated BV-2 cells*

The role of iNOS and COX-2 in inflammation was executed through NO and PGE_2, respectively. We further determined whether tripchlorolide could inhibit LPS-induced iNOS and COX-2 mRNA and protein expressions in BV-2 cells. Initial Western blot analysis revealed the expressions of iNOS and COX-2 in a time-dependent manner with a maximum at 12 h (data not shown). As shown in Fig. 6, both RT-PCR and Western blot analysis demonstrated that BV-2 cells expressed only a small amount of iNOS and COX-2. Upon LPS treatment (1 μg/ml) for 12 h, the expressions of mRNA and protein of iNOS and COX-2 in BV-2 cells were significantly increased (2.3-, 2.6-fold for iNOS and COX-2 mRNA, 2.5-, 4.8-fold for iNOS and COX-2 protein, respectively). However, the pretreatment of cells with tripchlorolide (1.25–10 nM) markedly attenuated the expressions of iNOS and COX-2 in both mRNA and protein in a concentration-dependent manner (Fig. 6). The maximal inhibitory potency of tripchlorolide at 10 nM was 59% for iNOS mRNA, 68% for COX-2 mRNA 74% for iNOS protein, and 56% for COX-2 protein (Fig. 6B and D). When tripchlorolide was at 1.25 nM, however, there were no significant changes in iNOS mRNA and protein expressions. Additionally, the inhibitory profile of tripchlorolide examined here on iNOS and COX-2 overlapped with its inhibitory activity on NO and PGE_2 production (Fig. 4A), respectively.

4. Discussion

The present study firstly demonstrated that tripchlorolide, an extract of the traditional Chinese herb *T. wilfordii*, protected the survival of primary cortical neurons and neuronal-like Neuro-2A cells from the neuroinflammatory damages induced by LPS-activated microglia. It also found that tripchlorolide significantly decreased LPS-induced inflammatory mediators and inhibited the production of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in both mRNA and

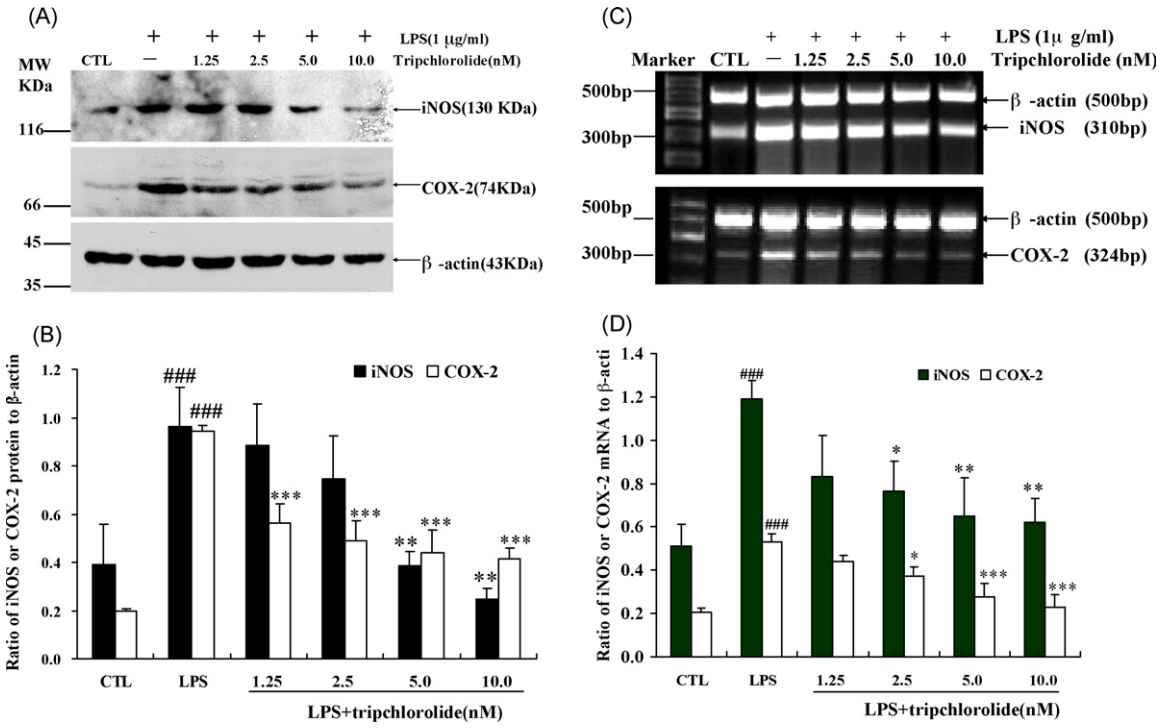

Fig. 6 – Effects of tripchlorolide (T4) on the expressions of iNOS and COX-2 mRNA and protein in BV-2 cells following LPS exposure. BV-2 cells were pretreated for 1 h with or without T4 (1.25–10 nM) and then were stimulated with LPS (1 μg/ml) for 12 h. (A) Representative of Western blot images revealed the expressions of iNOS and COX-2 protein. β-Actin was used as a control of protein loading. (C) Representative of RT-PCR image revealed the expressions of iNOS and COX-2 mRNA. β-Actin was used as an endogenous reference. (B and D) All the bands of Western blot or RT-PCR were scanned and ratios of optical density of specific bands to β-actin were illustrated. The values are expressed as mean ± S.D. of at least three independent experiments. $^{###}P < 0.001$, compared with control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with LPS treated alone group.

protein levels in microglial cells, providing an underlying mechanism for the neuroprotective effects of tripchlorolide *in vitro*.

Previously, Li et al. firstly reported that treatment with tripchlorolide (10^{-13} to 10^{-6} M) promoted neurite elongation with a pronounced effect at 10^{-13} M in primary culture of mesencephalic neurons [12]. In our study, tripchlorolide in a range of 5–80 nM was tested in microglial cells (primary microglia and BV-2 microglial cells) and neuronal cells (primary cortical neurons and Neuro-2A cells). Intriguingly, this agent (20–80 nM) was found to reduce the survival rate of microglial cells (Fig. 2) and induced apoptotic damage (data not shown), but had no cytotoxic action on primary cortical neurons. In fact, tripchlorolide (10–80 nM) significantly prolonged the survival of primary cultured cortical neurons. This effect appeared noticeably in 10 days *in vitro* in cultured cortical neurons (unpublished observation). These suggested that tripchlorolide may possess neurotrophic effects on primary cortical neurons.

A recent study has shown that triptolide protected DA neurons from microglia-mediated damage induced by LPS [9]. The current study revealed the conditioned media from LPS-stimulated microglial cells was highly toxic to neuronal cells, which was similar to Flavin et al. report [30]. In contrast, the survival ratio of neuronal cells was dramatically improved when they were treated with tripchlorolide prior to LPS stimulation in microglia. Therefore, it is reasonable to speculate that tripchlorolide may largely exert its neuroprotective effects via attenuating the abnormal elevation inflammatory mediators.

The choice of tripchlorolide dosage in this study was based on the following considerations. This study revealed that at a dose of 10 nM or lower, tripchlorolide did not exert a neurotoxic effect on either primary microglia or BV-2 cells (Fig. 2). When at a concentration of 10 nM, it exerted a significant neuroprotective effect against microglia-dependent LPS neurotoxicity in both primary cortical neurons and Neuro-2A cells (Fig. 3B–D) and when at a concentration of -10^{-8} M, a maximal neuroprotective effect against MPP+ neurotoxicity in primary culture of mesencephalic neurons [12]. Therefore, in order to minimize the potential side-effects of tripchlorolide, we chose the doses of 1.25, 2.5, 5.0 and 10 nM to investigate the anti-inflammatory effects of tripchlorolide in the above experiments.

The exposure of microglia cells to LPS elicited a wide spectrum of proinflammatory mediators, including TNF-α, IL-1β, NO and PGE_2 in the present study. These mediators stimulated by LPS have been shown to result in neuronal injury [15,31,32]. TNF-α is capable of inducing neuronal death when presented to neurons as part of a glial derived

inflammatory milieu [14,33,34]. IL-1β is known to be the major microglial signal that promotes the cascade of glial cell reactions [35]. NO plays the neurotoxic role in neuronal cell death, both *in vivo* [13,36] and *in vitro* [37,38]. Selective and non-selective inhibitors of NOS have resulted in significant neuroprotection [39,40]. COX-2 is markedly upregulated by inflammatory stimuli or growth factors and its reaction products have critical roles in a wide variety of pathological process related to glutamate excitotoxicity, including cerebral ischemia, and neurodegeneration [41]. COX-2-derived PGE_2 mediated the neurotoxicity via EP1 receptors, which are linked with Ca^{2+} [42]. Taken together, abundant evidence has demonstrated that the accumulation of inflammatory mediators contributes to neurodegeneration. Therefore, any compounds that inhibit microglia-mediated inflammation may be beneficial to the survival of neurons in brain.

Previous studies have demonstrated a strong anti-inflammatory activity of triptolide (PG490) on primary microglia [43], astrocyte [44] and monocyte [45]. *In vitro* PG490 inhibits inflammatory mediators including cytokine (IL-1β, TNF-α, and IL-6), NO and PGE_2 [44,46]. Moreover, triptolide inhibits LPS-induced microglial activation and attenuates the production of IL-1β and TNF-α in LPS injured tissue [9]. The present study demonstrated tripchlorolide, a structural analogue of triptolide, as a novel agent to suppress inflammatory process in central nervous system. It broadly inhibited LPS-induced the production of inflammatory mediators in microglial cells. The lipophilic character, together with its small molecular size (MW397), makes it a promising candidate for the treatment and/or prevention of neuroinflammation diseases *in vivo*. In addition, tripchlorolide at a concentration as low as 1.25–10 nM already had potent anti-inflammatory action. Meanwhile, in the range of this dose, it possesses neuroprotective role in neuronal cells damaged by microglia-mediated inflammation. This anti-inflammatory profile of tripchlorolide is similar to that of the previously mentioned anti-inflammatory drugs such as NF-κB inhibitors, which are reported to cause general repression of inflammatory mediators [47] upregulated by LPS. The suppression of either the activation and/or translocation of nuclear factor NF-κB results in the depression of proinflammatory cytokines production and iNOS expression. In fact, the mechanisms of triptolide inhibition of cytokine gene expression involve nuclear inhibition of transcriptional activation of NF-κB and the purine-box regulator operating at the antigen receptor response element (ARRE)/nuclear factor of activated T-cells (NF-AT) site after specific DNA binding [45].

LPS can induce intracellular SOA generation in microglial cells [19]. Accumulation of intracellular ROS triggered the release of inflammatory mediators in microglia via NF-κB pathway [10,26]. NF-κB is the key transcription factor that governs the expression of genes encoding proinflammatory cytokines and enzymes related to the inflammatory process, including IL-1β, TNF-α, iNOS and COX-2 [47]. Therefore, our finding that tripchlorolide attenuated LPS-induced SOA level suggests that blocking the induction of inflammatory mediators by tripchlorolide may involve ROS scavenging in LPS-activated microglia. However, due to the autocrine/paracrine regulatory effect of inflammatory mediators on microglia, proinflammatory cytokines and enzymes (e.g., COX-2 and iNOS) can also induce overproduction of intracellular reactive oxygen species (ROS) and NO [48–50]. Given the result of the present study that tripchlorolide markedly attenuated the expressions of iNOS and COX-2 in both mRNA and protein, thereby, we cannot rule out the probability of tripchlorolide reducing the intracellular ROS production via inhibiting the expressions of iNOS and COX-2 in LPS-activated microglia. Furthermore, no evidence has been shown that tripchlorolide interfered with LPS binding or other downstream sites. Therefore, more studies will be needed to clarify the mechanism of tripchlorolide in LPS-activated microglia.

In the AD brain, increased levels of TNF-α, iNOS and the peroxynitrite marker, nitrotyrosine, have been reported [51,52]. This elevation may be due to the activation of microglia. Although LPS was used to activate microglia in this study, tripchlorolide also suppressed the neurotoxic effectors of microglia stimulated with $A\beta_{1-42}$ (unpublished observation).

In summary, the present study demonstrated that tripchlorolide at nanomolar concentrations exerts potent anti-inflammatory effect on LPS-stimulated microglial cells and protects neuronal cells against neuroinflammatory toxicity induced by LPS-activated microglia. As the extract of TWHF is now widely used to treat patients with a variety of inflammatory and autoimmune diseases, such as rheumatoid arthritis in China, this drug may be useful in the treatment of many neurodegenerative disorders, such as AD, PD, multiple sclerosis and ischemic disorders in which microglial activation is a critical step in pathogenesis.

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REFERENCES

[1] Giulian D, Haverkamp LJ, Li J, Karshin WL, Yu J, Tom D, et al. Senile plaques stimulate microglia to release a neurotoxin found in Alzheimer brain. Neurochem Int 1995;27:119–37.
[2] Walker DG, Lue LF. Investigations with cultured human microglia on pathogenic mechanisms of Alzheimer's disease and other neurodegenerative diseases. J Neurosci Res 2005;81:412–25.
[3] McGeer PL, Kawamata T, Walker DG, Akiyama H, Tooyama I, McGeer EG. Microglia in degenerative neurological disease. Glia 1993;7:84–92.
[4] McGeer PL, McGeer EG. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Brain Res Rev 1995;21:195–218.
[5] Rich JB, Rasmusson DX, Folstein MF, Carson KA, Kawas C, Brandt J. Nonsteroidal anti-inflammatory drugs in Alzheimer's disease. Neurology 1995;45:51–5.
[6] Schenk D. Amyloid-beta immunotherapy for Alzheimer's disease: the end of the beginning. Nat Rev Neurosci 2002;3:824–8.
[7] Tao X, Cush JJ, Garret M, Lipsky PE. A phase I study of ethyl acetate extract of the Chinese antirheumatic herb

Tripterygium wilfordii Hook F in rheumatoid arthritis. J Rheumatol 2001;28:2160–7.
[8] Tao XL. [Mechanism of treating rheumatoid arthritis with *Tripterygium wilfordii* hook. II. Effect on PGE2 secretion]. Zhongguo Yi Xue Ke Xue Yuan Xue Bao 1989;11:36–40.
[9] Zhou HF, Liu XY, Niu DB, Li FQ, He QH, Wang XM. Triptolide protects dopaminergic neurons from inflammation-mediated damage induced by lipopolysaccharide intranigral injection. Neurobiol Dis 2005;18:441–9.
[10] Xue B, Jiao J, Zhang L, Li KR, Gong YT, Xie JX, et al. Triptolide upregulates NGF synthesis in rat astrocyte cultures. Neurochem Res 2007;32:1113–9.
[11] Yu DQ, Zhang DM, Wang HB, Liang XT. [Structure modification of triptolide, a diterpenoid from *Tripterygium wilfordii*]. Yao Xue Xue Bao 1992;27:830–6.
[12] Li FQ, Cheng XX, Liang XB, Wang XH, Xue B, He QH, et al. Neurotrophic and neuroprotective effects of tripchlorolide, an extract of Chinese herb *Tripterygium wilfordii* Hook F, on dopaminergic neurons. Exp Neurol 2003;179:28–37.
[13] Hong Z, Wang G, Gu J, Pan J, Bai L, Zhang S, et al. Tripchlorolide protects against MPTP-induced neurotoxicity in C57BL/6 mice. Eur J Neurosci 2007;26: 1500–8.
[14] Chao CC, Hu S. Tumor necrosis factor-alpha potentiates glutamate neurotoxicity in human fetal brain cell cultures. Dev Neurosci 1994;16:172–9.
[15] Le W, Rowe D, Xie W, Ortiz I, He Y, Appel SH. Microglial activation and dopaminergic cell injury: an in vitro model relevant to Parkinson's disease. J Neurosci 2001;21:8447–55.
[16] Giulian D, Baker TJ. Characterization of ameboid microglia isolated from developing mammalian brain. J Neurosci 1986;6:2163–78.
[17] Watters JJ, Sommer JA, Pfeiffer ZA, Prabhu U, Guerra AN, Bertics PJ. A differential role for the mitogen-activated protein kinases in lipopolysaccharide signaling: the MEK/ERK pathway is not essential for nitric oxide and interleukin 1beta production. J Biol Chem 2002;277: 9077–87.
[18] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 1982;126:131–8.
[19] Bruce-Keller AJ, Keeling JL, Keller JN, Huang FF, Camondola S, Mattson MP. Antiinflammatory effects of estrogen on microglial activation. Endocrinology 2000;141:3646–56.
[20] Choi HS, Kim JW, Cha YN, Kim C. A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. J Immunoassay Immunochem 2006;27:31–44.
[21] Huang Q, Wu LJ, Tashiro S, Onodera S, Ikejima T. Elevated levels of DNA repair enzymes and antioxidative enzymes by (+)-catechin in murine microglia cells after oxidative stress. J Asian Nat Prod Res 2006;8:61–71.
[22] Zhu YG, Chen XC, Chen ZZ, Zeng YQ, Shi GB, Su YH, et al. Curcumin protects mitochondria from oxidative damage and attenuates apoptosis in cortical neurons. Acta Pharmacol Sin 2004;25:1606–12.
[23] Pinteaux E, Rothwell NJ, Boutin H. Neuroprotective actions of endogenous interleukin-1 receptor antagonist (IL-1ra) are mediated by glia. Glia 2006;53:551–6.
[24] Taylor DL, Jones F, Kubota ES, Pocock JM. Stimulation of microglial metabotropic glutamate receptor mGlu2 triggers tumor necrosis factor alpha-induced neurotoxicity in concert with microglial-derived Fas ligand. J Neurosci 2005;25:2952–64.
[25] Wen W, Sanelli T, Ge W, Strong W, Strong MJ. Activated microglial supernatant induced motor neuron cytotoxicity is associated with upregulation of the TNFR1 receptor. Neurosci Res 2006;55:87–95.
[26] Min KJ, Jou I, Joe E. Plasminogen-induced IL-1beta and TNF-alpha production in microglia is regulated by reactive oxygen species. Biochem Biophys Res Commun 2003;312:969–74.
[27] Blasi E, Barluzzi R, Bocchini V, Mazzolla R, Bistoni F. Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. J Neuroimmunol 1990;27:229–37.
[28] Bocchini V, Mazzolla R, Barluzzi R, Blasi E, Sick P, Kettenmann H. An immortalized cell line expresses properties of activated microglial cells. J Neurosci Res 1992;31:616–21.
[29] Mirzoeva S, Koppal T, Petrova TV, Lukas TJ, Watterson DM, Van Eldik LJ. Screening in a cell-based assay for inhibitors of microglial nitric oxide production reveals calmodulin-regulated protein kinases as potential drug discovery targets.. Brain Res 1999;844:126–34.
[30] Flavin MP, Coughlin K, Ho LT. Soluble macrophage factors trigger apoptosis in cultured hippocampal neurons. Neuroscience 1997;80:437–48.
[31] Castano A, Herrera AJ, Cano J, Machado A. Lipopolysaccharide intranigral injection induces inflammatory reaction and damage in nigrostriatal dopaminergic system. J Neurochem 1998;70:1584–92.
[32] Gayle DA, Ling Z, Tong C, Landers T, Lipton JW, Carvey PM. Lipopolysaccharide (LPS)-induced dopamine cell loss in culture: roles of tumor necrosis factor-alpha, interleukin-1beta, and nitric oxide.. Brain Res Dev Brain Res 2002;133:27–35.
[33] Gelbard HA, Dzenko KA, DiLoreto D, del Cerro C, del Cerro M, Epstein LG. Neurotoxic effects of tumor necrosis factor alpha in primary human neuronal cultures are mediated by activation of the glutamate AMPA receptor subtype: implications for AIDS neuropathogenesis. Dev Neurosci 1993;15:417–22.
[34] Combs CK, Karlo JC, Kao SC, Landreth GE. Beta-Amyloid stimulation of microglia and monocytes results in TNFalpha-dependent expression of inducible nitric oxide synthase and neuronal apoptosis. J Neurosci 2001;21: 1179–88.
[35] Schubert P, Rudolphi K. Interfering with the pathologic activation of microglial cells and astrocytes in dementia. Alzheimer Dis Assoc Disord 1998;12(Suppl. 2):S21–8.
[36] Iravani MM, Kashefi K, Mander P, Rose S, Jenner P. Involvement of inducible nitric oxide synthase in inflammation-induced dopaminergic neurodegeneration. Neuroscience 2002;110:49–58.
[37] Lee P, Son D, Lee J, Kim YS, Kim H, Kim SY. Excessive production of nitric oxide induces the neuronal cell death in lipopolysaccharide-treated rat hippocampal slice culture. Neurosci Lett 2003;349:33–6.
[38] Jeohn GH, Kim WG, Hong JS. Time dependency of the action of nitric oxide in lipopolysaccharide-interferon-gamma-induced neuronal cell death in murine primary neuron-glia co-cultures. Brain Res 2000;880:173–7.
[39] Munch G, Gasic-Milenkovic J, Dukic-Stefanovic S, Kuhla B, Heinrich K, Riederer P, et al. Microglial activation induces cell death, inhibits neurite outgrowth and causes neurite retraction of differentiated neuroblastoma cells. Exp Brain Res 2003;150:1–8.
[40] Bal-Price A, Brown GC. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. J Neurosci 2001;21:6480–91.
[41] Minghetti L. Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. J Neuropathol Exp Neurol 2004;63:901–10.
[42] Kawano T, Anrather J, Zhou P, Park L, Wang G, Frys KA, et al. Prostaglandin E2 EP1 receptors: downstream effectors of COX-2 neurotoxicity. Nat Med 2006;12:225–9.

[43] Zhou HF, Niu DB, Xue B, Li FQ, Liu XY, He QH, et al. Triptolide inhibits TNF-alpha, IL-1 beta and NO production in primary microglial cultures. Neuroreport 2003;14:1091–5.
[44] Dai YQ, Jin DZ, Zhu XZ, Lei DL. Triptolide inhibits COX-2 expression via NF-kappa B pathway in astrocytes. Neurosci Res 2006;55:154–60.
[45] Qiu D, Zhao G, Aoki Y, Shi L, Uyei A, Nazarian S, et al. Immunosuppressant PG490 (triptolide) inhibits T-cell interleukin-2 expression at the level of purine-box/nuclear factor of activated T-cells and NF-kappaB transcriptional activation. J Biol Chem 1999;274:13443–50.
[46] Li FQ, Lu XZ, Liang XB, Zhou HF, Xue B, Liu XY, et al. Triptolide, a Chinese herbal extract, protects dopaminergic neurons from inflammation-mediated damage through inhibition of microglial activation. J Neuroimmunol 2004;148:24–31.
[47] Karin M, Yamamoto Y, Wang QM. The IKK NF-kappa B system: a treasure trove for drug development. Nat Rev Drug Discov 2004;3:17–26.
[48] Haddad JJ, Safieh-Garabedian B, Saade NE, Kanaan SA, Land SC. Chemioxyexcitation (delta pO2/ROS)-dependent release of IL-1 beta. IL-6 and TNF-alpha: evidence of cytokines as oxygen-sensitive mediators in the alveolar epithelium. Cytokine 2001;13:138–47.
[49] Haddad JJ. Recombinant human interleukin (IL)-1 beta-mediated regulation of hypoxia-inducible factor-1 alpha (HIF-1 alpha) stabilization, nuclear translocation and activation requires an antioxidant/reactive oxygen species (ROS)-sensitive mechanism. Eur Cytokine Netw 2002;13:250–60.
[50] Philipp S, Cui L, Ludolph B, Kelm M, Schulz R, Cohen MV, et al. Desferoxamine and ethyl-3,4-dihydroxybenzoate protect myocardium by activating NOS and generating mitochondrial ROS. Am J Physiol 2006;290:H450–7.
[51] Hensley K, Maidt ML, Yu Z, Sang H, Markesbery WR, Floyd RA. Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. J Neurosci 1998;18:8126–32.
[52] Tarkowski E, Blennow K, Wallin A, Tarkowski A. Intracerebral production of tumor necrosis factor-alpha, a local neuroprotective agent, in Alzheimer disease and vascular dementia. J Clin Immunol 1999;19:223–30.