

Triptolide, a Chinese herbal extract, protects dopaminergic neurons from inflammation-mediated damage through inhibition of microglial activation

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Abstract

Mounting lines of evidence have suggested that brain inflammation participates in the pathogenesis of Parkinson's disease. Triptolide is one of the major active components of Chinese herb *Tripterygium wilfordii* Hook F, which possesses potent anti-inflammatory and immunosuppressive properties. We found that triptolide concentration-dependently attenuated the lipopolysaccharide (LPS)-induced decrease in [³H]dopamine uptake and loss of tyrosine hydroxylase-immunoreactive neurons in primary mesencephalic neuron/glia mixed culture. Triptolide also blocked LPS-induced activation of microglia and excessive production of TNF α and NO. Our data suggests that triptolide may protect dopaminergic neurons from LPS-induced injury and its efficiency in inhibiting microglia activation may underlie the mechanism. © 2003 Elsevier B.V. All rights reserved.

Keywords: Triptolide; *Tripterygium wilfordii* Hook F; Inflammation; Microglia; Parkinson's disease; Neurodegeneration

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by cardinal features, including resting tremor, slowness of movement, rigidity, and postural instability as a result of progressive loss of the dopaminergic neurons in substantia nigra pars compacta (SNpc). The cause responsible for the progressive loss of dopaminergic neurons is largely unknown. Recent evidence suggests glia-mediated inflammation is involved in this process. Activation of microglia in the SNpc has been demonstrated in human patients or experimental

animals exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (Hirsch, 2000; Langston et al., 1999; McGeer et al., 1988). Under normal conditions, microglial cells, as the resident macrophages in the brain, remain at a low immunoreactive state and serve as immune surveillance (Kreutzberg, 1996). In response to abnormal stimulation, such as neurotoxins, neuronal debris, or brain trauma, microglia cells become active very rapidly. Activated microglia are believed to contribute to neurodegeneration through the release of cytotoxic compounds, including reactive oxygen intermediates, nitric oxide (NO), proteases and pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin 1 beta (IL-1 β) (Bal-Price and Brown, 2001; McGeer and McGeer, 1995).

Extracts of Chinese herb *Tripterygium wilfordii* Hook F (TWHF), which is commonly called Lei-Gong-Teng in China, have been found to have potent anti-inflammatory and immunosuppressive properties and are used widely in China for treatment of rheumatoid arthritis (Tao, 1989). Triptolide (designated as T₁₀) is one of the major active ingredients of TWHF, which is a diterpene triepoxide and structurally analogous to another active ingredient, triptochlorolide (Fig. 1). Our previous work shows that triptochlorolide reduced the toxicity of 1-methyl-4-phenyl-

Abbreviations: DA, dopamine; FBS, fetal bovine serum; ICC, immunocytochemistry; IL, interleukin; LPS, lipopolysaccharide; MEM, minimum essential medium; MFB, medial forebrain bundle; MPP⁺, 1-methyl-4-phenylpyridinium ion; NF- κ B, nuclear factor-kappa B; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PD, Parkinson's disease; ROS, reactive oxygen species; TNF α , tumor necrosis factor- α ; SNpc, substantia nigra pars compacta; TWHF, *Tripterygium wilfordii* Hook F.

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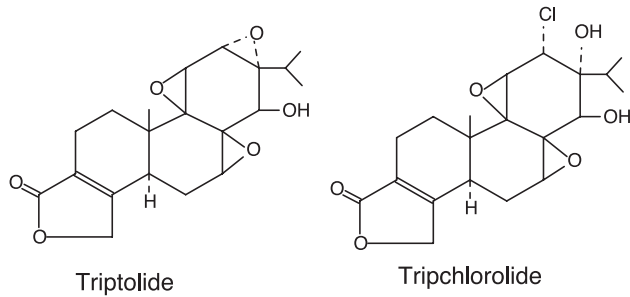


Fig. 1. The chemical structures of triptolide (T_{10}) and tripchlorolide (TW397).

pridinium ion (MPP^+) and prevented degeneration of dopaminergic neurons induced by axotomy of medial fore-brain bundle (MFB) (Li et al., 2003). Most recently, we found that tripchlorolide potently attenuated elevation of $TNF\alpha$ levels in the brain of MFB-axotomized rats (Cheng et al., 2002). It has been well documented that both tripchlorolide and triptolide possess potent anti-inflammatory and immunosuppressive effects (Zhao et al., 2000; Zheng et al., 1994). Therefore, we speculate that T_{10} may attenuate the inflammatory responses of microglial cells to exogenous toxins and consequently protect inflammation-mediated degeneration of dopaminergic neurons. In this study, this hypothesis was evaluated by using an endotoxin lipopolysaccharide (LPS). LPS-induced dopaminergic degeneration that mimics most of the phenomena and process of inflammatory dopaminergic degeneration has been proven to be a successful model to screen potential compounds for therapy of PD (Le et al., 2001).

2. Methods

2.1. Reagents

Triptolide (98% in purity) was provided by Prof. Pengfei Tu, Peking University Pharmaceutical School. Cell culture reagents were obtained from Invitrogen (Carlsbad, CA) and [3H]dopamine (DA) (30 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). The monoclonal antibody against the CR3 complement receptor (OX-42) was obtained from Chemicon (Temecula, CA). The monoclonal anti-tyrosine hydroxylase (TH) antibody was purchased from Sigma (St. Louis, MO). Rat $TNF\alpha$ detection ELISA kits were purchased from Endogen (Woburn, MA). A Vectastain ABC kit and biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA).

2.2. Rat mesencephalic neuron-glia cultures

Embryonic mesencephalic neuron-glia cultures were obtained from timed pregnant Sprague–Dawley rats on embryonic day 14 (E14). Briefly, the ventral mesencephalic

tissues were removed and dissociated to single cells by a mild mechanical trituration. Cells were seeded at 5×10^5 /well to 24-well culture plates pre-coated with poly-D-lysine (20 $\mu\text{g}/\text{ml}$) and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in a maintenance medium. The medium consists of minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 10% heat-inactivated horse serum (HS), 1 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin. Seven-day-old cultures were used for the treatment. The composition of the cultures at the time of treatment was approximately 45% astrocytes, 13% microglia, 42% neurons and $\sim 1\%$ TH-immunoreactive (IR) neurons.

2.3. [3H]DA uptake assay

[3H]DA uptake assays were performed as previously described (Liu et al., 2000a). Briefly, cultures were incubated for 20 min at 37 °C with 1 μM [3H]DA in Krebs-Ringer buffer (KRB, 16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl_2 , 1.2 mM MgSO_4 , 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4). After washing three times with ice-cold KRB, cells were lysed in 1 N NaOH. Radioactivity was determined by Packard liquid scintillation analyzer (TRI-CARB 2300TR, Packard BioScience, USA). Non-specific DA uptake observed in the presence of mazindol (10 μM) was subtracted.

2.4. Tyrosine hydroxylase (TH)-immunocytochemistry and morphological analysis

Dopaminergic neurons were recognized with anti-TH antibody and microglia was detected with OX-42 antibody, which recognizes the CR3 receptor as previously described (Liu et al., 2000a). Briefly, formaldehyde (3.7%)-fixed cultures were treated with 1% hydrogen peroxide for 10 min followed by sequential incubation with blocking solution for 30 min, primary antibody (overnight, 4 °C), biotinylated secondary antibody (2 h), and ABC reagents (40 min). Color was developed with 3,3'-diaminobenzidine (DAB). For morphological analysis, images were recorded with a phase contrast microscope (DMIRB, Leica) equipped with SPOT-2 digital camera (Diagnostic Instruments, USA) and MetaMorph image processing and analysis software (Universal Imaging, USA). For visual counting of TH-IR neurons, nine representative areas per well of the 24-well plate were counted under the microscope at 100 \times magnification.

To measure neurite length of TH-IR neurons, immunostained neurons were randomly photographed for 20 fields per well at 200 \times magnification. Lengths of axons (defined as the longest process per neuron) were measured with MetaMorph image processing and analysis software. Only processes over 2 \times cell body size were measured. Data

obtained from identically treated wells (two per group) were pooled and histograms were constructed.

2.5. Nitrite assay

The production of NO was determined by measuring the accumulated levels of nitrite in supernatants by the Griess reaction. To measure nitrite, 50- μ l aliquots were removed from supernatants of cultured cells and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride and 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined in a Bio-Rad microplate reader. The concentration of nitrite was calculated using sodium nitrite as a standard.

2.6. Quantification of TNF α by ELISA

TNF α secretion was measured by solid-phase ELISA technique (Endogen). Briefly, a 96-well ELISA plate was pre-coated with hamster anti-mouse monoclonal TNF α capture antibody overnight and 50- μ l sample or mouse TNF α standards were added to each well. Following incubation for 2 h at room temperature, the wells were washed, after which goat anti-mouse TNF α secondary horseradish peroxidase conjugate was added and incubated for another 2 h. Following two washes, tetramethylbenzidine solution was added, and the peroxidase-catalyzed color change was stopped by acidification with 2 N H_2SO_4 . The plate was scanned at $\lambda=450$ nm and the absorbance was measured with a Bio-Rad microplate reader.

2.7. Statistical analysis

Data were expressed as the mean \pm SEM. Statistical significance was assessed with an analysis of variance (ANOVA) followed by Newman–Keul's post hoc test using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of T_{10} on LPS-induced degeneration of dopaminergic neurons

Mesencephalic neuron-glia mixed cultures were pretreated with vehicle or T_{10} (1–10 nM) 30 min prior to application of 5 ng/ml of LPS. Seven days later, the degeneration of dopaminergic neurons was assessed by [3H]DA uptake or TH immunostaining. [3H]DA uptake assays showed that treatment with LPS reduced the uptake capacity by approximately 40% of control group. T_{10} , at the concentration of 1, 5, and 10 nM, exhibited concentration-dependen-

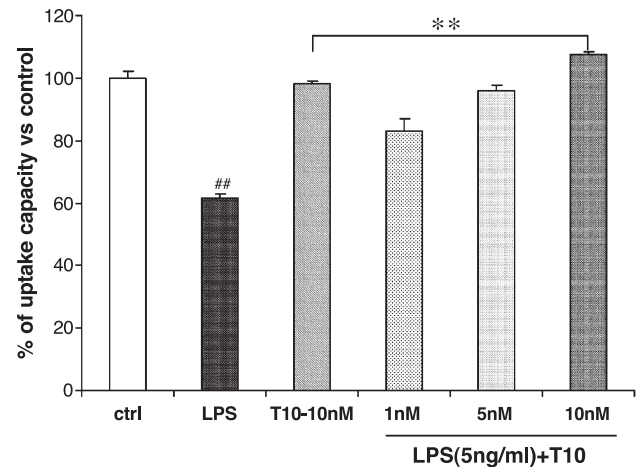


Fig. 2. Effect of T_{10} on LPS-induced degeneration of dopaminergic neurons on mesencephalic neuron-glia cultures. Cultures were treated with vehicle, or indicated concentrations of T_{10} 30 min prior to treatment with 5 ng/ml LPS. Seven days later, neurotoxicity was assessed by [3H]DA uptake as described in Materials and methods. Data are expressed as percentage of the control group and presented as mean \pm SEM of three experiments performed in triplicate. ##, $p < 0.01$ compared with control, and **, $p < 0.01$ compared with the group of LPS-treated alone.

dent protection by 21%, 34% and 46%, respectively, compared with that of LPS-treated alone (Fig. 2). The most effective concentration was observed at 10 nM, which provided a complete protection against LPS-mediated damage. DA uptake of cultures treated with 10 nM T_{10} alone did not differ significantly from that of control cultures (vehicle-treated only), suggesting T_{10} was devoid of obvious toxicity (Fig. 2).

Counting the number of TH-IR neurons in the cultures revealed that LPS reduced the number of TH-IR neurons by 49% as compared to vehicle-treated control cultures (Fig. 3E), whereas only 22% and 3% loss of TH-IR neurons was observed when treated with T_{10} at the concentration of 1 and 10 nM, respectively (Fig. 3E). Morphologically, in addition to reduction in abundance, the remaining TH-IR neurons in the LPS-treated cultures had a significantly fewer dendrites, shorter or even truncated axons (Fig. 3B,F). In cultures pretreated with T_{10} (10 nM), changes in the TH-IR dendrites were less profound as compared with that of LPS-treated cultures (Fig. 3B). In the cultures treated with T_{10} (10 nM) alone, no obvious damage to TH-IR neurons was observed (Fig. 3C) and it appeared there were more TH-IR neurons with longer neurites than those seen in the vehicle-treated control cultures (Fig. 3E,F).

3.2. Effects of T_{10} on LPS-induced microglial activation

To elucidate the underlying mechanism of the neuroprotective activity of T_{10} , we investigated the effect of T_{10} on the LPS-induced microglial activation revealed by OX-42 immunostaining and nitric oxide assay. Under normal conditions, microglial cells stay in a resting state and

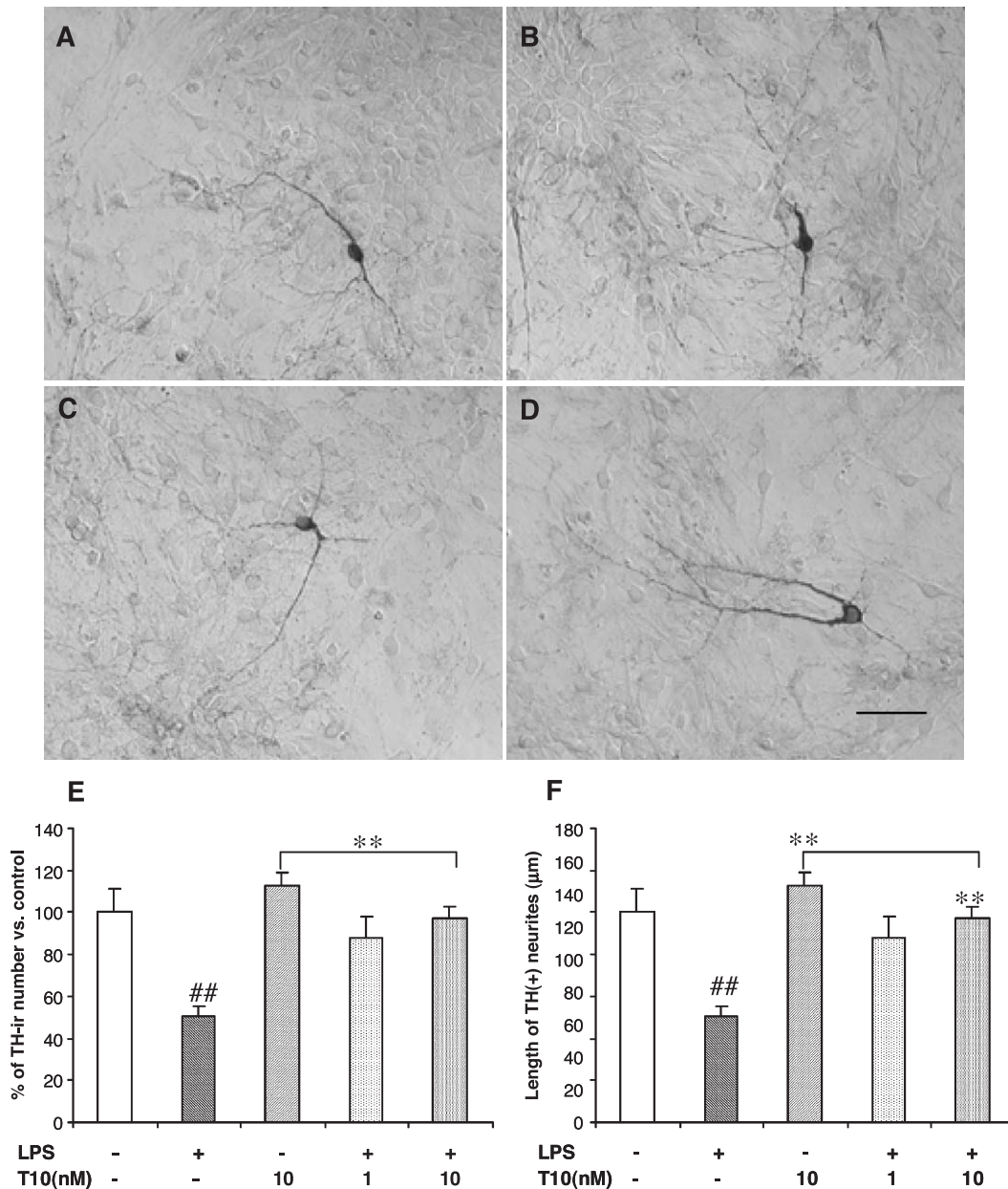


Fig. 3. Morphological evidences of the protective effect of T_{10} against LPS-induced damage to dopaminergic neurons in mesencephalic neuron-glia cultures. After maintaining in maintenance medium for 7 days, cultures were changed with treatment medium and treated with vehicle or different concentrations of T_{10} 30 min prior to treatment with 5 ng/ml LPS. TH immunocytochemistry was performed to reveal dopaminergic neurons after treatment for 7 days. Images were captured with SPOT-2 cooled CCD combined with MetaMorph software. (A) Control group (vehicle alone); (B) LPS (5 ng/ml) alone; (C) T_{10} (10 nM) alone; (D) T_{10} (10 nM) with LPS. The magnification is $200\times$ and bar = 250 μm . (E) The total number of TH-IR neurons in each well was counted under converted microscope and data were presented as mean \pm SEM ($n=3$). (F) Neurite length of TH-positive neurons were also measured by MetaMorph software and data were presented as mean \pm SEM. ##, $p < 0.01$ compared with control group, and **, $p < 0.01$ compared with LPS-treated group.

morphologically display as non-ramified cells without processes and are relatively smaller in size (Fig. 4A). After treatment with LPS (5 ng/ml) for 20 h, OX-42 immunoreactive microglia showed a highly differentiated state with enlarged cell body and stout processes (Fig. 4B). Interestingly, treatment with T_{10} (10 nM) obviously blocked the LPS-induced activation of microglia (Fig. 4D). T_{10} alone (without LPS) showed no evidence to activate microglia (Fig. 4C).

Of the numerous neurotoxic factors, $\text{TNF}\alpha$ and NO may be major mediators of dopaminergic neurodegeneration elicited by LPS challenging (Gayle et al., 2002). Accumulation of nitrite, an indicator of LPS-stimulated production of NO, was determined at 24 and 48 h and $\text{TNF}\alpha$ was measured by ELISA at 3 h following LPS stimulation. As shown in Figs. 5 and 6, treatment with LPS produced robust increases in both $\text{TNF}\alpha$ and NO levels, and pretreatment with T_{10} potently reduced the increases in a concentration-dependent

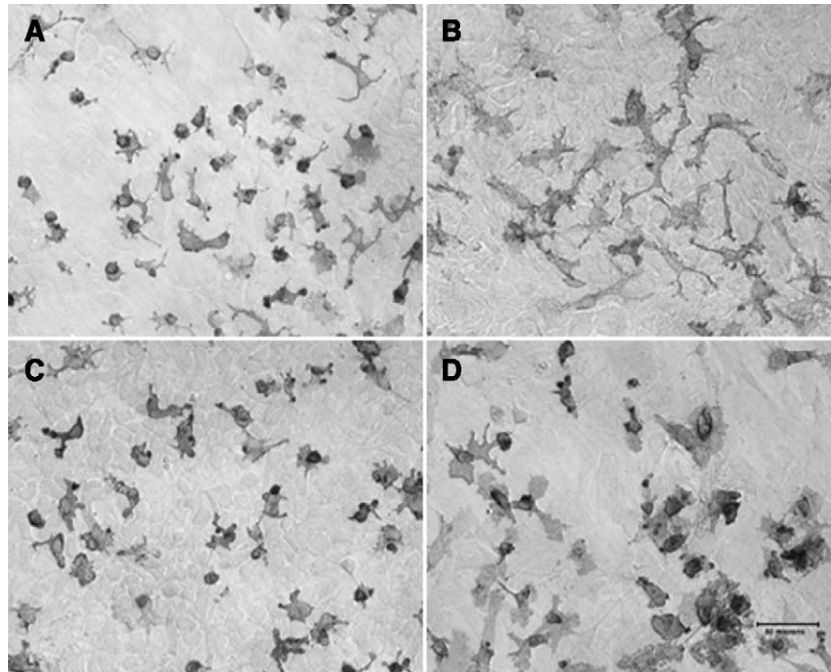


Fig. 4. Effect of T₁₀ on LPS-induced microglial activation in primary mesencephalic neuron-glia cultures. T₁₀ (10 nM) or vehicle was added 30 min prior to LPS (5 ng/ml) treatment. The cultures were fixed with 3.7% formaldehyde 21 h after LPS treatment for further OX-42 immunostaining, which is a biomarker of murine microglia. The images were captured under converted phase-contrast microscope equipped with SPOT-2 CCD at a magnification of 200 ×. The bar shown at the bottom of the left panel equals to 200 μm. (A) Control group (vehicle alone); (B) LPS-treated alone; (C) T₁₀ (10 nM)-treated alone; (D) T₁₀ (10 nM) with LPS.

fashion. T₁₀ at 5 and 10 nM attenuated the production of TNFα by 21% and 58%, respectively (Fig. 5). At the equivalent concentration, T₁₀ reduced the level of nitrite measured at 48 h after LPS stimulation by 37% and 57% as

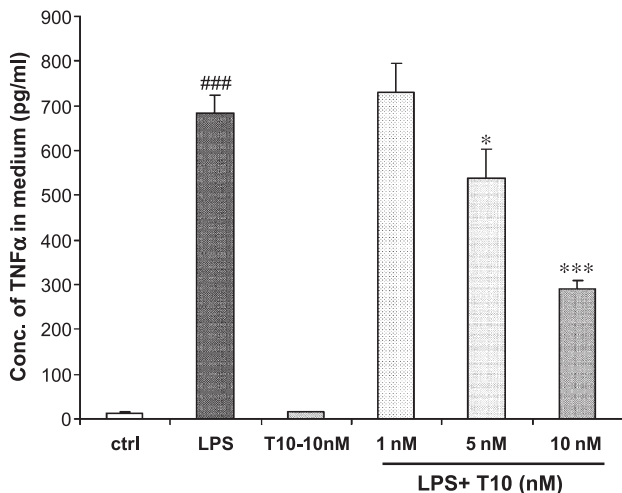


Fig. 5. Effect of T₁₀ on LPS-induced production of TNFα in primary mesencephalic cultures. Indicated concentrations of T₁₀ were applied 30 min prior to LPS (5 ng/ml) treatment, and 50 μl of supernatant per well was collected at 3 h for TNFα ELISA as described in Materials and methods. The experiments were repeated three times and the similar pattern of inhibition was observed. The data were presented as mean ± SEM (n=4). ###, p < 0.001 compared with control group and *, p < 0.05; ***, p < 0.001 compared with the group of LPS-treated alone.

compared to the culture of LPS-treated alone (Fig. 6). In addition, T₁₀ alone showed no significant effects on the production of TNFα and NO in primary neuron-glia cultures (Figs. 5 and 6).

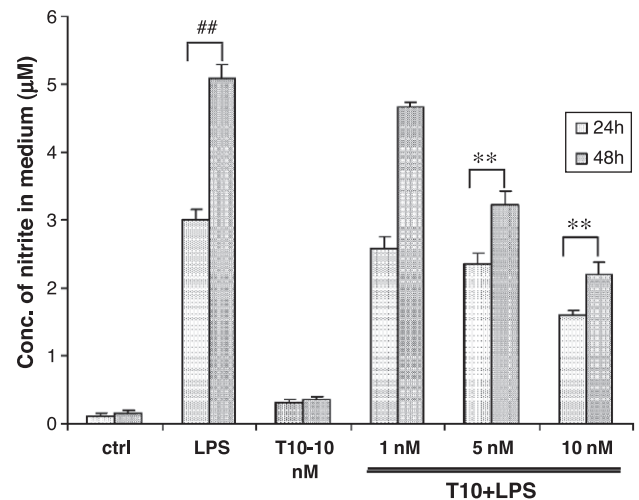


Fig. 6. Effect of T₁₀ on LPS-induced production of NO in primary mesencephalic cultures. T₁₀ was applied 30 min prior to LPS (5 ng/ml) treatment, and 50 μl of supernatant per well was collected at 24 and 48 h for NO assay. A representative result from three independent experiments is shown. The data were presented as mean ± SEM (n=4). ##, p < 0.01 compared with control group and **, p < 0.01 compared with the group of LPS-treated alone.

4. Discussion

In the mesencephalic mixed neuron-glia culture, stimulation of microglial cells with an inflammagen LPS induces the production of neurotoxic factors, including TNF α , interleukin 1-beta (IL-1 β), NO, superoxide, and consequent degeneration of dopaminergic neurons (Gayle et al., 2002). Therefore, the *in vitro* model of LPS-induced dopaminergic neurodegeneration provides a powerful tool for mechanistic studies and identification of potential therapeutic agents (Le et al., 2001). Using this model, we first report that triptolide, a diterpene triepoxide purified from the Chinese herbal medicine TWHF, potently protected dopaminergic neurons against LPS-induced degeneration. Moreover, the neuroprotective effect of T₁₀ may be associated with its ability to inhibit the production of neurotoxic factors, such as TNF α and NO, by LPS-activated microglia.

TWHF has been used for centuries in traditional Chinese medical remedies to “stimulate blood circulation, relieve stasis, reduce inflammation, relieve edema, purge excess internal warmth, and eliminate toxicity” (Li, 1578). T₁₀ is the first extract from TWHF described by Kupchan et al. (1972) and has been found to be the most potent ingredient of TWHF in anti-inflammatory and immunosuppressive actions (Zheng, 1991). Previous studies showed that T₁₀ significantly inhibited the LPS-induced TNF α production in mouse macrophage and bronchial epithelial cells (Lin et al., 2001). The production of NO was also inhibited by extracts of TWHF in a variety of cells (Guo et al., 2001). The present study showed that T₁₀ markedly blocked the activation of microglia as revealed by both morphological (Fig. 4) and biochemical (Figs. 5 and 6) methods. Our findings extend its anti-inflammatory spectrum and, together with its lipophilic property, strongly support the possibility for T₁₀ to exert anti-inflammatory action in the CNS, especially for the treatment of inflammation-mediated neurological diseases, such as PD and AD. Moreover, with triptolide as the major active ingredient of TWHF (Lei-Gong-Teng), Lei-Gong-Teng tablet has been approved by the Bureau of Chinese Medicine Administration to treat rheumatoid arthritis in China. The suggested safe and effective dose is at the range of 0.5–5 μ g/kg/day expressed as the active component, triptolide. This dose range is roughly equivalent to 1.5–15 nM *in vitro*. So, the concentrations (i.e., 1–10 nM) we used in the present study match with the clinical effective dose. This, in turn, strongly suggests that the neuroprotective effect observed in the present investigation is most probably derived from its anti-inflammatory property.

An increasing attention has been drawn to the neuro-immune hypothesis of PD's etiology (Vila et al., 2001). Several lines of evidence indicate that the dopaminergic system in the midbrain is most susceptible to inflammation-mediated damage (Vila et al., 2001). Microglia are suspected to be instrumental to this process. Recently, Kim et al. (2000) reported that the density of microglial cells is remarkably higher in the SN compared to other midbrain

areas and brain regions such as the hippocampus. Aside from dramatic loss of dopaminergic neurons, the SN is also the site of a glial reaction in both PD patients and experimental models of PD (McGeer et al., 1988; Kohutnicka et al., 1998). Although the question as to whether microglial activation is a primary event or is secondary to neuronal death, activated glial cells, especially microglia, can produce a variety of noxious compounds including reactive oxygen species, reactive nitrogen species, pro-inflammatory cytokines, etc. In particular, TNF α plays a critical role in promoting the infiltration of inflammatory cells, intracerebral immune responses and the production of other cytokines (Mogi et al., 1994). For example, TNF α has been implicated in enhanced microglial release of NO and other reactive oxygen species (Merrill et al., 1993). Moreover, excessive production of TNF α reversibly promotes further activation of microglia and astroglia, suggesting a positive feedback loop for TNF α expression. Therefore, limiting the initial production of TNF α is important for blocking the deleterious feedback loop. In the present study, T₁₀ exhibited such a limiting effect. This early interference with TNF α production may account for the neuroprotective action of T₁₀.

Among the array of reactive species secreted by active microglia, attention has been given to reactive nitrogen species due to the prevailing idea that nitric oxide-mediated nitrating stress could be pivotal in the pathogenesis of Parkinson's disease (Liberatore et al., 1999; Torreilles et al., 1999). Excessive accumulation of NO has long been known to be toxic to neurons (Bronstein et al., 1995; Jeohn et al., 2000). The overproduction of free radicals is especially deleterious to neurons (Floyd, 1999). However, when NO meets with superoxide, a more deadly nitrite, peroxynitrite, is formed, which is a potent oxidant and nitrating agent capable of attacking and modifying proteins, lipids and DNA as well as depleting antioxidant defenses (Torreilles et al., 1999; Estevez and Jordan, 2002). In fact, a recent study has identified peroxynitrite as a key mediator of neurotoxicity induced by LPS-activated microglia (Xie et al., 2002), a similar finding in this study that challenging microglia with LPS induced a robust production of both NO and superoxide. More interestingly, T₁₀ substantially inhibited NO production by 57% in neuron-glia cultures. This potency of NO inhibition matches the potent iNOS inhibitor G \ddot{o} 6976, which antagonizes LPS-induced degeneration of dopaminergic neurons *in vitro* (Jeohn et al., 2000). Like G \ddot{o} 6976, T₁₀ also blocked the LPS-induced iNOS activation in primary microglial cultures (Zhou et al., 2003). Given that iNOS is the prime isoform of NOS stimulating NO production in the brain after inflammatory assault (Liberatore et al., 1999; Iravani et al., 2002), the present result provides a reasonable interpretation to the inhibition of NO production by T₁₀ and consequent neuroprotection.

In the past several years, rapid progress has been made in understanding the molecular mechanism of the immunosuppressive and anti-inflammatory actions of T₁₀. Qiu et al. (1999) reported that the immunosuppressive property of T₁₀

involved nuclear inhibition of transcriptional activation of NF- κ B and the purine-box regulator which operates at the antigen receptor response element (ARRE)/nuclear factor of activated T cells (NF-AT) at a step after specific DNA binding. Now, T₁₀ has been thought as a specific inhibitor of NF- κ B (Liu et al., 2000b). Interestingly, increasing evidence suggests that NF- κ B may involve the etiology of Parkinson's disease, revealed by the evidence that robust increment of NF- κ B activation and nucleus translocation was found in postmortem study (Hunot et al., 1997), and in DA or MPP⁺-induced apoptosis of PC12 or SH-SY5Y (Panet et al., 2001). A large body of literature reveals that NF- κ B is activated rapidly in response to a wide range of pathways, including LPS, TNF α and T-cell receptor signaling (Li and Verma, 2002). In turn, activated NF- κ B can influence the expression of a large number of genes, including iNOS and TNF α (Mattson and Camandola, 2001). Especially for microglia, NF- κ B activation is ultimately important for strong induction of TNF α (Jana et al., 2002). Therefore, in combination with our findings in the present study, we speculate that the inhibitory effect of T₁₀ on NF- κ B activation may contribute to its neuroprotective action against LPS-elicited DA degeneration. Although the present study could not explain exactly how T₁₀ works, its ability to dampen the initial inflammatory cascade, including the production of TNF α and NO, is obviously beneficial to the survival of DA neurons.

In summary, our work showed a potent neuroprotective effect of Chinese herbal extract T₁₀ on dopaminergic neurons in the in vitro chronic inflammatory model. The anti-inflammatory property of T₁₀ contributes to its neuroprotective effect. As suggested by its efficacy in vitro, and because of its lipophilic nature, the therapeutic implications of T₁₀ may thus prove successful in slowing or even halting further degeneration of PD targeting a specific aspect of the glia-related cascade of deleterious events. Further study in the in vivo animal model is highly urgent to confirm its effectiveness.

Acknowledgements

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