Triptolide protects dopaminergic neurons from inflammation-mediated damage induced by lipopolysaccharide intranigral injection

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Converging lines of evidence suggest that neuroinflammatory processes may account for the progressive death of dopaminergic neurons in Parkinson's disease (PD). Therefore, anti-inflammatory strategies have attracted much interest for their potential to prevent further deterioration of PD. Our previous study showed that triptolide, a traditional Chinese herbal compound with anti-inflammatory and immunosuppressive properties, protected dopaminergic neurons from lipopolysaccharide (LPS)-induced damage in primary embryonic midbrain cell cultures. To examine further if triptolide can protect dopaminergic neurons from inflammation-mediated damage in vivo, microglial activation and injury of dopaminergic neurons were induced by LPS intranigral injection, and the effects of triptolide treatment on microglial activation and survival ratio and function of dopaminergic neurons were investigated. Our results demonstrated that microglial activation induced by a single intranigral dose of 10 μg of LPS reduced the survival ratio of tyrosine hydroxylase-immunoreactive (TH-ir) neurons in the substantia nigra pars compacta (SNpc) to 29% and the content of dopamine (DA) in striatum to 37% of the non-injected side. Intriguingly, treatment with triptolide of 5 μg/kg for 24 days once per day dramatically improved the survival rate of TH-ir neurons in the SNpc to 79% of the non-injected side. Meanwhile, treatment with triptolide of 1 or 5 μg/kg for 24 days once per day significantly improved DA level in striatum to 70% and 68% of the non-injected side, respectively. Complement receptor 3 (CR3) immunohistochemical staining revealed that triptolide treatment potently inhibited LPS-elicited deleterious activation of microglia in SNpc. The excessive production of cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, was significantly abolished by triptolide administration. These results, together with our previous data in vitro, highly suggest the effectiveness of triptolide in protecting dopaminergic neurons against inflammatory challenge.
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Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive death of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Although the mechanism of neuronal degeneration still remains to be uncovered, postmortem analysis and animal experiments have provided evidence for suggesting that neuroinflammatory processes may account for the progressive death of dopaminergic neurons (Grunblatt et al., 2001; Hunot and Hirsch, 2003). Postmortem studies have shown that there was a large number of reactive microglia in the SN in PD, particularly in areas of maximal neurodegeneration, namely, the ventral and lateral portion of the SN (Hirsch et al., 1998). A robust activation of microglia was also found in both 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)- and 6-hydroxydopamine (6-OHDA)-induced Parkinsonism animal models (Kitamura et al., 1994; Kurkowska-Jastrzebska et al., 1999; Matsuura et al., 1997).

Under normal physiological conditions, microglia are involved in immune surveillance and host defense against infectious agents. However, microglia readily become activated in response to injury or immunological challenges, as indicated by a change in morphology from a ramified resting state to an amoeboid appearance with an increase in the expression of complement receptor 3 (CR3). Activated microglia is believed to contribute to neurodegeneration through the release of cytotoxic compounds, such as proinflammatory cytokines, nitric oxide (NO), excitatory amino acids, reactive oxygen intermediates, and arachidonic acid and derivatives (Streit et al., 1999).

Castano and colleagues reported that, in contrast to the transient damage on serotoninergic system, the inflammatory reaction induced by lipopolysaccharide (LPS) intranigral injection had a irreversibly neurodegenerative effect on the dopaminergic neurons in both the cell body and the terminal areas during the entire 21-day studying period (Castano et al., 1998). This neurotoxicity differs from that of well-known neurotoxins such as 6-OHDA and 1-methyl-4-phenylpyridinium ion (MPP^+). 6-OHDA or MPP^+ induce significant dopaminergic dysfunction as early as 12–72 h postinjury that lasts for longer than a month (Jeon et al., 1995), whereas significant dopaminergic damage by LPS shows a delay. This difference could be due to a different mechanism of neurotoxicity, as 6-OHDA and MPP^+ kill neurons directly, but LPS acts via microglial activation and consequent release of toxic cytokines (Bronstein et al., 1995). As the SN contains the highest concentration of microglia in the rodent (Lawson et al., 1990), neurons in the SN are more sensitive to LPS-induced degeneration than those in other brain regions (Kim et al., 2000).

Given the role of microglia in mediating neurodegeneration, a great deal of effort has been made to develop novel treatments of PD by targeting microglia and associated inflammatory factors (Castano et al., 2002). In our recent effort in exploring new drugs, extracts of the traditional Chinese herb *Tripterygium wilfordii* Hook F (TWHF) have drawn our attention. The extract of TWHF has been reported to be effective in the treatment of a variety of inflammatory and autoimmune diseases, such as rheumatoid arthritis (Gu and Brandwein, 1998). Triptolide (designated as T_{10}) has been identified as the major component responsible for the immunosuppressive and anti-inflammatory effects of TWHF (Gu et al., 1995). Accumulating data have demonstrated a strong anti-inflammatory action of T_{10} on multiple tissues (Qiu and Kao, 2003; Zhao et al., 2000).

Our previous work revealed that tripchlorolide (TW397), a structural analogue of T_{10} (Tao and Lipsky, 2000; Zhang et al., 1993), protected dopaminergic neurons from the neurotoxic lesion induced by MPP^+ in embryonic mesencephalic neuronal cultures. In vivo administration of TW397 prevented neuronal cell death in the SNpc and depletion of dopamine in the striatum of medial forebrain bundle transected Parkinsonism model rats (Li et al., 2003). In order to clarify if the neuroprotective effect of T_{10} was mediated by its anti-inflammatory activity, we investigated the effect of T_{10} on LPS-induced damage to dopaminergic neurons in primary embryonic midbrain cell cultures. It was found that T_{10} concentration-dependently attenuated the LPS-induced decrease in [^{3}H]dopamine uptake and loss of TH-ir neurons in vitro (Li et al., 2003), and the neuroprotective action might result from the inhibition of LPS-induced microglial activation and the release of cytotoxic compounds (Zhou et al., 2003). To examine further if T_{10} can protect dopaminergic neurons from inflammation-mediated damage in vivo, microglial activation and injury of dopaminergic neurons were induced by LPS intranigral injection, and the effects of T_{10} treatment on microglial activation and the survival and function of dopaminergic neurons were investigated.

Materials and methods

Source of triptolide

Triptolide (T_{10}) was generously provided by Professor Peng-Fei Tu (the School of Pharmaceutical Sciences, Peking University). The white crystal is 98% pure by reverse phase high-performance liquid chromatography (HPLC).

Animals and surgery

Fifty-six adult male Wistar rats weighing 250–300 g were supplied by the Laboratory Animal Center, Peking University, and housed in a standard 12-h on/off light cycle with food and water ad libitum in the home cage. They were allowed to acclimate to their new surroundings for 10 days before experimental manipulation. The rats were anesthetized with 350 mg/kg chloral hydrate and positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, U.S.A.) to conform to the brain atlas of Paxinos and Watson (1986). LPS (from *Escherichia coli*, serotype O26:B6, Sigma, St. Louis, MO, U.S.A.) was dissolved (5 mg/ml) in phosphate-buffed saline (PBS), and 2.0 μl was injected into the right SNpc. The injection needle was lowered through a drill hole 5.3 mm posterior, 2 mm lateral, and 7.8 mm ventral to bregma. The injection was delivered over 2 min, and after each injection, the needle was left in situ for an additional 5 min to avoid reflux along the injection track. Thereafter, the skull surface was covered with fibrosponge and the skin was sutured. Sham-operated animals were subjected to the same surgical procedures except that 2 μl PBS was injected into the SNpc. The experimental procedures were approved by the Committee on Animal Care and Usage of Peking University Health Science Center, and all effort was made to minimize animal suffering.

Application of T_{10}

Rats were divided into four groups of thirteen to fifteen: the sham-operated group, the LPS-injected group followed by vehicle treatment, the LPS-injected group followed by treatment with 1 or 5 μg/kg T_{10}, respectively. T_{10} was resolved in physiological saline containing 5% dimethylsulphoxide and applied intraperitoneally. Rats received a single dose of 1 or 5 μg/kg T_{10} or vehicle injection at 8:00 A.M. once per day from 3 days before LPS injection up to 21 days post LPS injection (24 days in total).

Rotational behavior assay

Rats were placed into cylinders attached to a rotameter (Columbus Instruments, Columbus, OH, U.S.A.) to examine the rotational behavior induced by amphetamine on the second day after the final T_{10} injection. They were allowed to adapt for 10 min to the testing environment and then were injected intraperitoneally with 5 mg/kg D-amphetamine sulfate (Sigma, St. Louis, MO, U.S.A.) dissolved in physiological saline. Measurement of rotational activity began 5 min after injection and lasted for 45 min under minimal external stimuli. The lights were turned

off, and the room was sealed from noise. The rotameter recorded the number of full clockwise and counterclockwise turns the animals made during the testing period. Clockwise turns (ipsilateral to LPS injection) were counted as positive turns, whereas counterclockwise turns (contralateral to LPS injection) were counted as negative turns. The net number of turns made during the entire 45-min testing period were counted and divided by 45 to calculate the rotational rate.

Tissue collection and processing

On the second day after assay of rotational behavior, four rats from each group were randomly selected for morphological studies. All other rats were decapitated and the bilateral SN and striatum were dissected quickly and stored in $-80°C$ until analysis. The SN was used for the quantification of proinflammatory cytokines and the striatum for determination of DA content. For the morphological studies, rats were deeply anesthetized with chloral hydrate, and then transcardially perfused with 100 ml saline followed by 200 ml 4% paraformaldehyde in phosphate buffer. Brains were removed and postfixed in the same fixative and cryoprotected by 30% sucrose for 3–5 days. The brains were frozen on powdered dry ice and then arranged for frontal sectioning according to the rat brain atlas of Paxinos and Watson. Frozen sections (35 μm in thickness) were cut with a cryostat (Leica, Germany) at $-20°C$ and used for immunohistochemistry as described further below.

Quantification of DA and its metabolite, dihydroxyphenylacetic acid (DOPAC)

For these studies, striatum processed and stored at $-80°C$, as described above, was used. The contents of DA and its metabolite, dihydroxyphenylacetic acid (DOPAC), were determined using an HPLC apparatus with an electrochemical detector (Model 5600A CoulArray Detector System ESA, Brighton, MA, U.S.A.). Briefly, tissues were homogenized in 200 mM ice-cold perchloric acid. The homogenate was placed in an ice bath for 60 min. Subsequently,
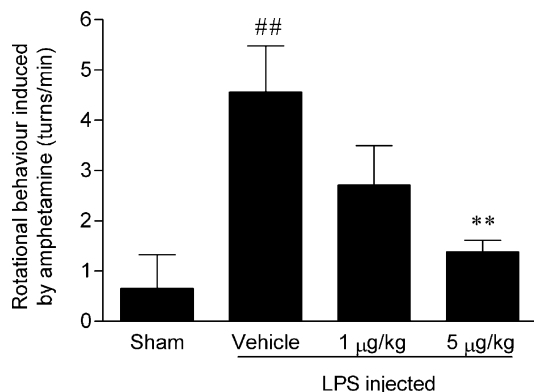

Fig. 1. Effect of T_{10} treatment on amphetamine-induced rotational behavior. Rats were randomly grouped and then pretreated with T_{10} (1 and 5 μg/day, i.p.) or vehicle 3 days before LPS injection and subsequently for 21 days after LPS injection (24 days in total). On day 25, rats were injected with D-amphetamine (5 mg/kg, i.p.) to challenge rotational behavior. Total turns were counted by computerized rotameter for 45 min and the rotational rate, expressed as turns per minute, was regarded as the index of degree of lesion. $n = 7–11$. $^{\#\#}P < 0.01$ versus the sham group. $^{**}P < 0.01$ versus the vehicle group.
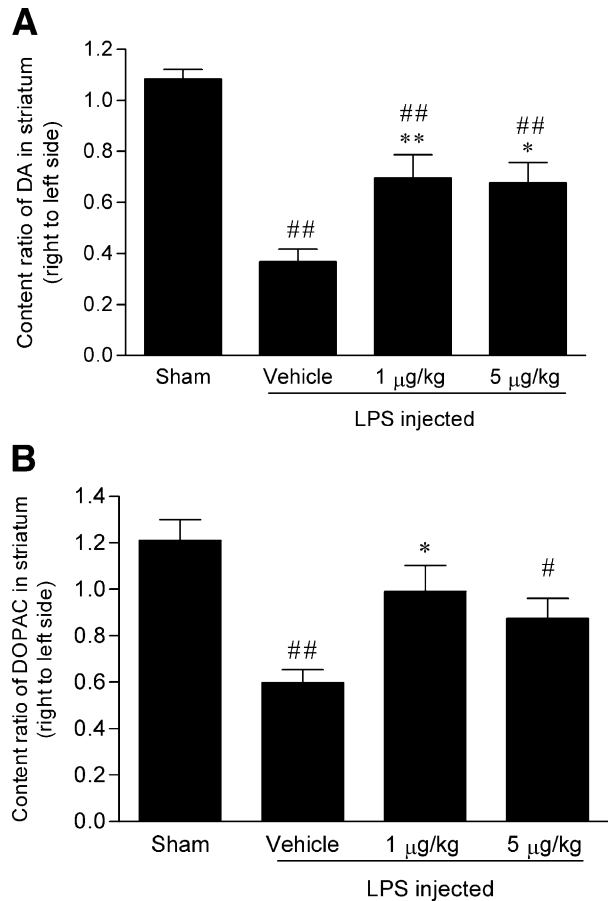

Fig. 2. Effect of T_{10} treatment on the content of DA and DOPAC in the striatum. Rats were randomly grouped and then pretreated with T_{10} (1 and 5 μg/day, i.p.) or vehicle 3 days before LPS injection and subsequently for 21 days after LPS injection (24 days in total). On day 25, rats were decapitated and the striatum were dissected. The content of DA (A) and DOPAC (B) in the striatum was detected by HPLC, and the ratio of right to left side was calculated. $n = 9–11$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the sham group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the vehicle group.

the sample was centrifuged at $15,000 \times g$ for 20 min at $4°C$ and the supernatant was transferred to a clean tube and measured for volume. One-half volume of a solution containing 20 mM potassium citrate, 300 mM potassium dihydrogen phosphate, and 2 mM EDTA·2Na was added and mixed in thoroughly to deposit perchloric acid. After incubating in an ice bath for 60 min, the mix was centrifuged at $15,000 \times g$, for 20 min at $4°C$. The supernatant was filtered through a 0.22-μm Millipore filter and then injected into the HPLC system for analysis. The mobile phase was 125 mM sodium citrate buffer containing 20% methanol, 0.1 mM EDTA·2Na, 0.5 mM 1-octanesulfonic acid sodium salt (Acros Organics, Morris Plains, NJ, U.S.A.) adjusted to pH 4.3. The flow rate was set at 1.2 ml/min. Striata from nine to eleven animals in each treatment group were used.

Immunohistochemistry and quantification of dopaminergic neuronal survival rate and microglial activation

All sections spanning the SN (bregma -4.80 mm to -6.30 mm) were collected and used for immunohistochemistry. Every sixth section through the compacta region was processed for
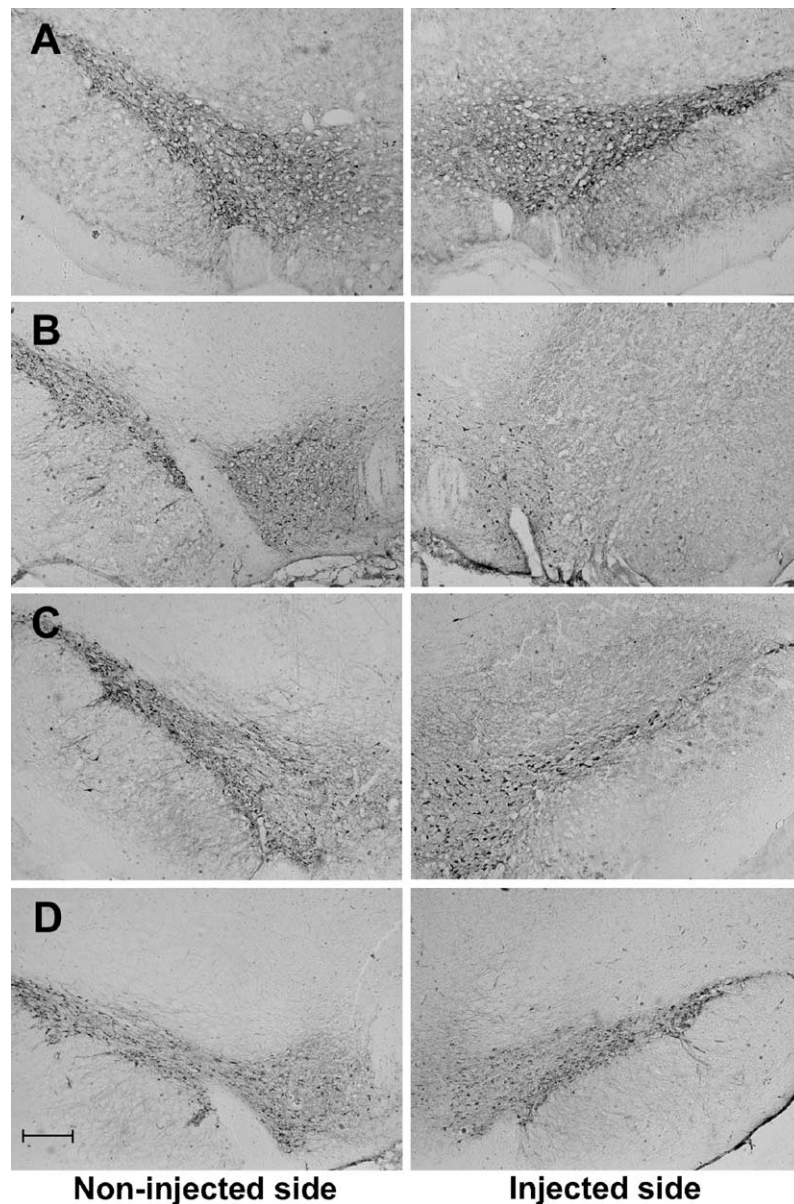
Non-injected side   **Injected side**

Fig. 3. Morphological evidence of the protective effect of T_{10} against LPS-induced damage to dopaminergic neurons in the SN. Rats were randomly grouped and then pretreated with T_{10} (1 and 5 μg/day, i.p.) or vehicle 3 days before LPS injection and subsequently for 21 days after LPS injection (24 days in total). On day 25, rats were transcardially perfused by 4% paraformaldehyde. Frozen sections (35 μm in thickness) were cut and TH was detected by immunohistochemical staining to show dopaminergic neurons in the SNpc. The photographs were captured by the imaging analysis system (SPOT-2). (A) Photograph demonstrating that in sham-operated rats, TH staining is similar on the non-injected (left) and PBS-injected (right) sides; (B) Twenty-one days after the LPS injection, a profound loss of TH-ir cells is seen on the injected side in the animals treated with vehicle after LPS injection; (C and D) In rats treated with 1 μg/kg (C) or 5 μg/kg (D) T_{10} for 24 days, more TH-ir cells survived on the injected side compared to the injected side of the vehicle-treated rats. Scale bar represents 100 μm.

immunohistochemical detection of the dopaminergic neuronal marker tyrosine hydroxylase (TH, Sigma, St. Louis, MO, U.S.A.). Adjacent sections were immunostained for detection of the monocyte/macrophage/microglial marker CR3 (OX-42 or CD11b, Chemicon, Temecula, CA, U.S.A.). The anti-TH and anti-CR3 mouse monoclonal antibodies were used at 1:3300 and 1:400 dilutions, respectively. Sections were incubated with primary antibodies for 24 h at 4°C. Inclusion of diluted non-immune goat serum instead of the primary antibodies served as negative controls. Sections were incubated in biotinylated goat anti-mouse antibody and then in the avidin–biotin–peroxidase

complex for 30 min at 37°C. The bound complex was visualized by incubating sections in a solution containing 0.1% 3, 3ʹ-diaminobenzidine (Sigma, St. Louis, MO, U.S.A.), 1% H_2O_2, and 8% ammonium nickel sulfate (Fluka Chemie GmbH, Switzerland).

TH-ir neurons with distinct nuclei were counted in six sections throughout the entire rostrocaudal extent of the SNpc. All sections were coded and examined blind. The survival rate of dopaminergic neurons in the SNpc was determined by counting the number of TH-ir neurons on LPS-injected side relative to the number of TH-ir neurons on the non-injected side.

The CR3-stained sections were used to establish the extent of microglial activation in the SNpc. The outline of the compacta region was determined in the adjacent TH-stained sections by the distribution of the dopaminergic neurons and the well-established landmarks. The SNpc on both the injected and non-injected side were analyzed blind as to the animal treatment using an advanced image-analysis system (Metmorph). Acquisition of the average optical density (OD) was accomplished by using a $40\times$ objective under normal bright-field illumination (Olympus, Japan). Data were resolved relative to a 255-level gray scale and the value was converted to OD units. Background values derived from areas where positive signal was absent were subtracted from the OD values. Six sections from each animal were recorded.

Quantification of proinflammatory cytokines

For these studies, we used the SN, processed and stored at $-80°C$ as described above. Tissues were homogenized in ice-cold tissue lysis buffer containing 137 mM NaCl, 20 mM Tris (pH 8.0), 1% (v/v) Glycerol, 1% (v/v) Nonidet P-40 (NP40), 1 mM phenyl-methylsulfonyl fluoride (PMSF), and 0.5 mM sodium vanadate. The homogenate was centrifuged at $1500 \times g$ for 15 min at $4°C$. The supernatant was collected and kept at $4°C$. If not used immediately, the supernatant was stored at $-80°C$ until assay. TNF-α and IL-1β levels were detected by rat TNF-α and IL-1β enzyme-linked immunoadsorbent assay (ELISA) kits (Pierce Biotechonology Inc., Rockford IL, U.S.A.), respectively, according to the procedures provided by the manufacturer. The sensitivity of the assay was 10 pg/ml for TNF-α ELISA and 12 pg/ml for IL-1β ELISA. The standards were performed in duplicate. The protein concentration was determined using a detergent-compatible protein assay with a bovine serum albumin standard. Substantia nigra tissue from seven animals in each treatment group was used.

Statistical analysis

Data were processed by commercially available software GraphPad Prism 3.0. Results are typically presented as means ± SEM. Statistical significance was assessed using a one-way ANOVA followed by Dunnett post hoc test (compares all the other columns with the designated control column). Significance was set at $P < 0.05$.

Results

T_{10} administration improves post-surgical recovery

Compared with vehicle treatment after LPS intranigral injection, T_{10} treatment substantially improved the recovery from LPS injection. Within 8 h, T_{10}-treated rats were actively moving around the cage, showing a normal feeding and drinking behavior, without other signs of post-surgical stress. T_{10}- but not vehicle-treated rats were able to maintain their body weight during the entire period of T_{10} application.

T_{10} administration improves functional recovery from LPS intranigral injection

As mentioned above, LPS intranigral injection leads to microglial activation and injury of dopaminergic neurons in the SN. Administration of amphetamine, an indirect agonist of DA receptor, elicits rotational behavior towards the injection side. The rotational rate of sham-operated animals was 0.66 ± 1.76 turns/min. Vehicle-treated control animals examined at 21 days after LPS injection exhibited higher rotational rate than sham-operated animals ($P < 0.01$). However, treatment with T_{10} (5 μg/kg) for 24 days significantly reduced the amphetamine-induced rotational behavior (1.38 ± 0.77 vs. 4.56 ± 3.04 turns/min, $P < 0.01$) (Fig. 1). A low dose (1 μg/kg) of T_{10} exhibited a trend toward lower rotational rate, although not statistically significant.

T_{10} administration attenuates depletion of DA and DOPAC in the striatum induced by LPS intranigral injection

The adequacy of the LPS injection was confirmed by a decrease of striatal DA content. In the vehicle-treated control group, the levels of DA and DOPAC on the LPS-injected side were reduced to 37% and 60% of the non-injected side, respectively. Treatment with T_{10} (1, 5 μg/kg, i.p.) for 24 days significantly attenuated DA depletion in the striatum induced by LPS intranigral injection (Fig. 2). The levels of DA on the LPS-injected side were 70% and 68% of the non-injected side in animals treated by 1 and 5 μg/kg T_{10}, respectively (Fig. 2A). The levels of DOPAC on the LPS-injected side were 99% and 87% of the non-injected side in groups treated with 1 and 5 μg/kg T_{10}, respectively (Fig. 2B).

T_{10} treatment protects dopaminergic neurons from the injury induced by LPS intranigral injection

In sham-operated animals, the number of TH-ir neurons was similar on the ipsilateral and contralateral side to the injection (Fig. 3A); the survival ratio of TH-ir neurons was 90% (Fig. 4). Animals that received vehicle treatment after LPS intranigral injection showed a marked loss of TH-ir neurons and their dendrites (Fig. 3B); only 29% TH-ir neurons in the SNpc on the LPS-injected side survived, compared with those on the non-
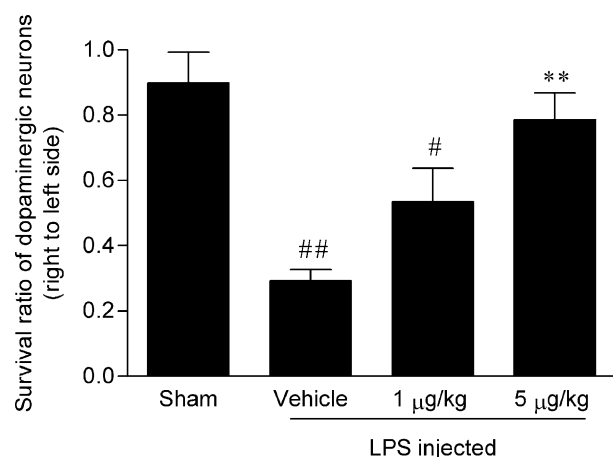

Fig. 4. Effect of T_{10} treatment on the survival ratio of dopaminergic neurons in the SNpc. TH-ir neurons in the SNpc were counted as described in Materials and methods. Survival ratio of the dopaminergic neurons in the SNpc (the injected side versus the non-injected side) was calculated. $n = 4$. [#]$P < 0.05$, [##]$P < 0.01$ versus the sham group. **$P < 0.01$ versus the vehicle group.
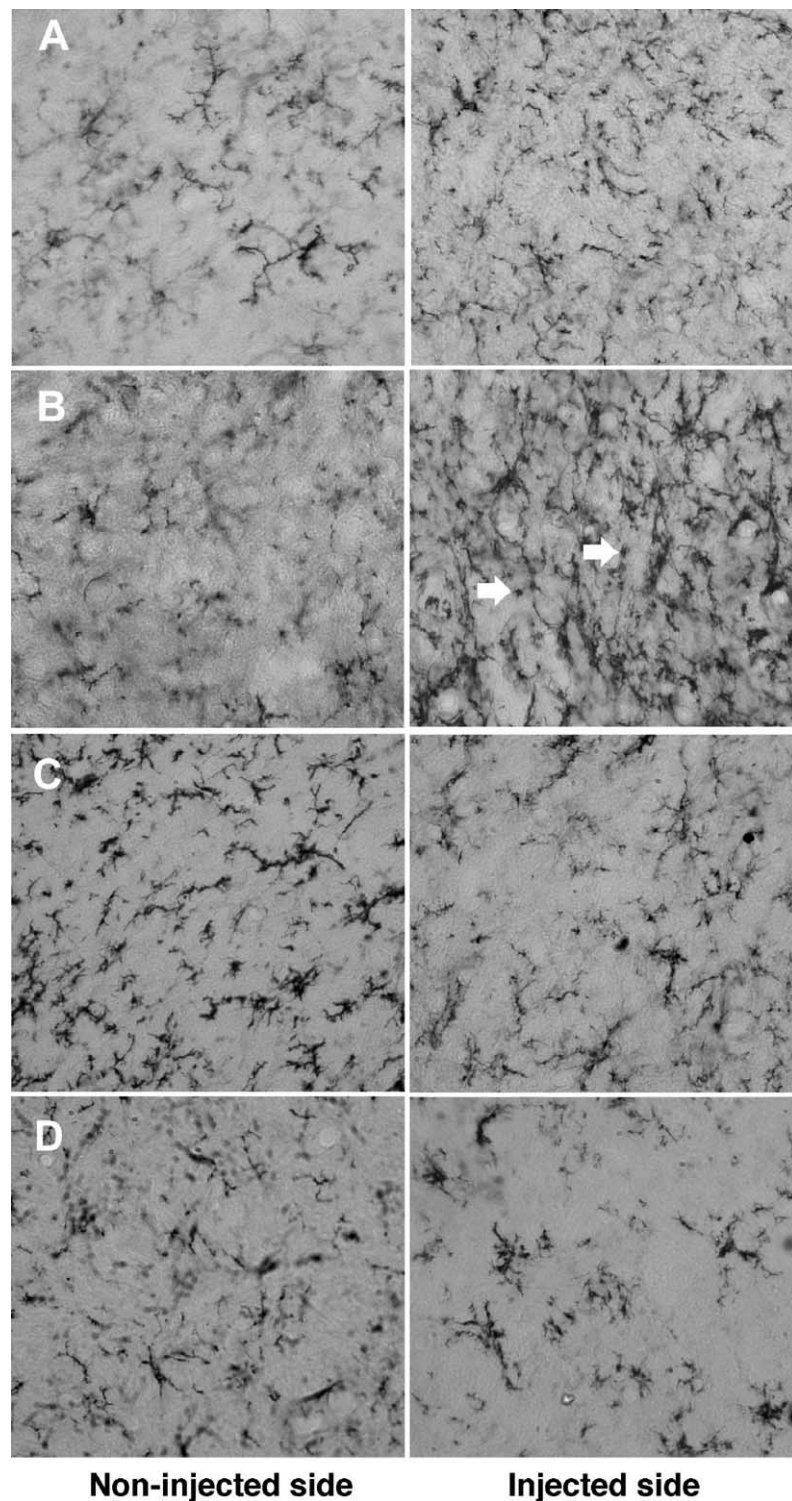
Non-injected side   **Injected side**

Fig. 5. Morphological evidence of the inhibitory effect of T_{10} treatment on microglial activation induced by LPS intranigral injection. Rats were randomly grouped and then pretreated with T_{10} (1 and 5 μg/day, i.p.) or vehicle 3 days before LPS injection and subsequently for 21 days after LPS injection (24 days in total). On day 25, rats were transcardially perfused by 4% paraformaldehyde. Frozen sections (35 μm in thickness) were cut and CR3 was detected by immunohistochemical staining to show the microglia in the SN. The photographs were captured by the imaging analysis system (SPOT-2). (A) CR3-ir cells in PBS-injected (sham) SN were typically resident microglia with two or three fine processes; (B) 21 days after LPS injection, CR3-ir cells showed activated morphology with shorter and thicker processes than resident cells. Besides microglial cells, cells of macrophage morphology stained by CR3 can be seen (indicated by arrows); (C and D) After having been treated with 1 μg/kg (C) or 5 μg/kg (D) T_{10} for 24 days, activation of microglial cells was inhibited obviously. Scale bar represents 200 μm.
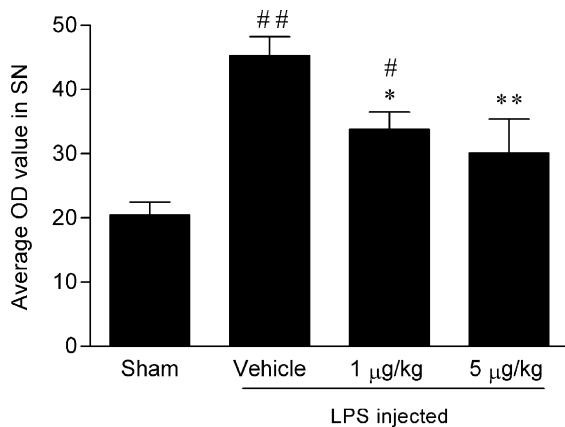
Fig. 6. Effects of T_{10} treatment on the average optic density of CR3 in the SN. The outlines of the compacta region were determined by the distribution of dopaminergic neurons in the adjacent TH-stained sections. $n = 4$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the sham group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the vehicle group.

injected side (Fig. 4). In contrast, a significant sparing of the TH-ir neurons was observed in the animals treated with T_{10} and the dendritic processes surrounding the TH-ir neurons were largely preserved (Fig. 3D). T_{10} at the dose of 5 μg/kg preserved as many as 79% TH-ir cells on the LPS-injected side, compared with those on non-injected side (Fig. 4). T_{10} at the dose of 1 μg/kg was less effective, showing a preservation of 53% without statistical significance to the LPS-injected vehicle-treated group (Figs. 3C, 4).

T_{10} treatment inhibits microglial activation induced by LPS intranigral injection

CR3-ir cells were ramified resting microglia with two or three fine processes in the SNpc of sham-operated animals (Fig. 5A). After local injection of LPS with vehicle treatment, activated microglia were readily identifiable throughout the SNpc by their thicker processes and more rounded cell body. In addition, many amoeboid microglia could be found in the SNpc. (Fig. 5B). After LPS injection plus treatment with 1 (Fig. 5C) or 5 (Fig. 5D) μg/kg T_{10}, the activation of microglia was inhibited significantly, more microglia in these groups remained in the ramified morphology than in the vehicle-treated control group. As shown in Fig. 5, no significant change was found on the non-injected side of the above groups. On the injected side, the content of CR3 was increased significantly in the LPS-injected vehicle-treated control ($P < 0.01$). After treatment with 1 and 5 μg/kg T_{10} for 24 days, the content of CR3 was significantly reduced in the SNpc by 25.4% and 33.6%, respectively (Fig. 6).

Focal injection of LPS induced excessive release of proinflammatory cytokines. In the SN of sham-operated animals and on the non-injected side of LPS-injected animals, the concentrations of TNF-α and IL-1β were below the detection limit of the commercial ELISA kits. In LPS-injected vehicle-treated control, the concentrations of TNF-α and IL-1β in the SN were 1.27 ± 0.52 pg/mg protein and 9.29 ± 3.50 pg/mg protein, respectively. Moreover, treatment with 1 μg/kg T_{10} significantly reduced the concentration of TNF-α and IL-1β by 64.6% and 43.5%, respectively. Treatment with 5 μg/kg T_{10} reduced the production of TNF-α and IL-1β by 73.2% and 65.1%, respectively (Fig. 7).

Discussion

Our results demonstrated in vivo that microglial activation induced by a single intranigral dose of 10 μg of LPS has a degenerative effect on the dopaminergic neurons in the nigrostriatal system. T_{10}, a traditional Chinese herbal compound with anti-inflammatory and immunosuppressive properties, significantly improved the behavioral manifestation of dopaminergic degeneration and prevented the loss of dopaminergic neurons. We also found that T_{10} potently inhibited LPS-induced activation of microglia and production of deleterious cytokines, which may underlie the mechanism of the neuroprotective effect of T_{10}.

The extract of TWHF has been reported to be effective in the treatment of a variety of inflammatory and autoimmune diseases, such as rheumatoid arthritis (Cibere et al., 2003; Gu and Brandwein, 1998). T_{10} has been identified as the major component responsible for the immunosuppressive and anti-inflammatory effects of TWHF (Gu et al., 1995). Accumulating data has demonstrated a strong anti-inflammatory activity of T_{10} on monocytes, fibroblasts, and bronchial epithelial cells. In vitro experiments demonstrated that triptolide inhibited prostaglandin E2 production in monocytes and fibroblasts by blocking the up-regulation of cyclooxygenase 2 (Tao et al., 1998). Moreover, triptolide inhibited human bronchial epithelial cell expression of IL-6 and IL-8 stimulated by phorbol 12-myristate 13-acetate
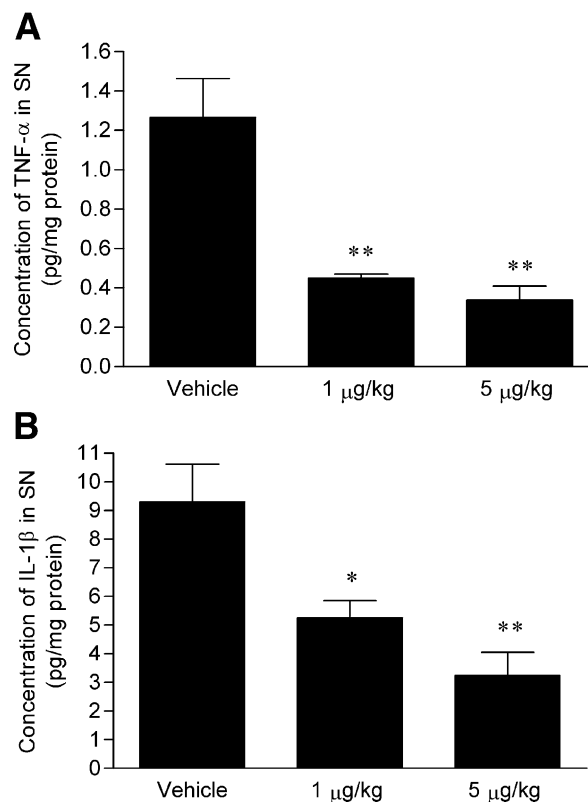

Fig. 7. Effects of T_{10} treatment on the concentrations of proinflammatory cytokines in the SN of LPS-injected rats. Rats were randomly grouped and then pretreated with T_{10} (1 and 5 μg/day, i.p.) or vehicle 3 days before LPS injection and subsequently for 21 days after LPS injection (24 days in total). On day 25, rats were decapitated and the SN were dissected. The concentration of TNF-α (A) and IL-1β (B) was detected by commercial ELISA kit. $n = 7$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the vehicle group.

(PMA), TNF-α, or IL-1β. Nuclear runoff and luciferase reporter gene assays demonstrated that triptolide inhibits IL-8 transcription (Zhao et al., 2000). In vivo experiments showed that treatment with triptolide resulted in a delay of onset of arthritis, as well as decreased arthritis incidence and arthritis severity and in vivo cell-mediated immunity to collagen (Gu and Brandwein, 1998).

Since T_{10} passes through the blood–brain barrier easily for its lipophilic character and its small molecular size (MW 360) (Zhang and Deng, 1991), we studied its potential anti-inflammatory effect in the central nervous system (CNS). Our previous work demonstrated that treatment with T_{10} dose-dependently reduced the LPS-induced nitrite accumulation and TNF-α and IL-1β release in primary cultured microglia. T_{10} treatment reduced LPS-stimulated mRNA expression of all three inflammatory factors (Zhou et al., 2003). Moreover, our present in vivo data showed that T_{10} inhibited microglial activation in the SN induced by LPS intranigral injection, indicated by the changes in microglial morphology and CR3 expression and proinflammatory cytokine levels in the SN. Taken together, both our in vitro and in vivo experiments indicated that T_{10} had a potent anti-inflammatory activity in the CNS.

It was well documented that neuroinflammation, characterized by microglial activation, may be involved in the degeneration of dopaminergic neurons in PD. Microglia are the main immune cells in the CNS. Under normal physiological conditions, resting microglia are evenly scattered throughout the brain. They have small cell bodies and fine ramified processes. Microglia can become activated following neuronal injury induced by either axotomy or administration of neurotoxins. Upon activation, microglia undergo specific morphological changes such as increase in cell body size, a shortening and thickening of processes, and an amoeboid appearance. These modifications are accompanied by immunophenotypic changes characterized by increased expression of CR3, as determined by OX-42 immunostaining (Kreutzberg, 1996; Streit et al., 1999). Meanwhile, activated microglia release a variety of cytotoxic mediators, including NO, proinflammatory cytokines, excitatory amino acids, reactive oxygen intermediates, and arachidonic acid and its derivatives (Streit et al., 1999). Most evidence indicates that proinflammatory cytokines may mediate neuronal degeneration through the activation of iNOS and subsequent high release of NO from the rodent glial cells (Dugas et al., 2001; Hunot et al., 1999).

LPS, a component of the gram-negative bacterial cell wall, is a potent inducer of inflammation. LPS treatment can activate microglia in vitro to release proinflammatory cytokines, such as TNF-α and IL-1β (Le et al., 2001; Liu et al., 2000a). In the current study, we showed that focal injection of LPS induced microglial activation and release of proinflammatory cytokines in the SN (Figs. 5, 6, and 7). Interestingly, in LPS-injected rats, the administration of amphetamine, an indirect agonist of DA receptor, elicited rotational behavior towards the side ipsilateral to the injection. Furthermore, there was a significant decrease in the number of TH-ir cells in SNpc and the depletion of DA and its metabolite DOPAC in striatum. These results are in agreement with previous studies showing that LPS intranigral injection induces an inflammatory reaction and damage in nigrostriatal dopaminergic system (Castano et al., 1998; Liu et al., 2000b). Moreover, it has been demonstrated that neurons in the SN are more sensitive to LPS-induced neurotoxicity than neurons in hippocampus or cortex, presumably resulting from its higher concentration of microglia than that of other brain regions (Kim et al., 2000). Thus, in our experiments, LPS intranigral injection led to the local degeneration of dopaminergic neurons in the SN and it represents a good model for PD-like neurodegeneration and motor deficits.

Our previous work revealed that compounds of TWHF had potent neuroprotective effect on the dopaminergic neurons both in vitro and in vivo. It was demonstrated that TW397 protected dopaminergic neurons from the neurotoxic lesion induced by MPP^+ in embryonic mesencephalic neuronal cultures. In vivo administration of TW397 prevented neuronal cell death in the SNpc and depletion of dopamine in the striatum of medial forebrain bundle transected Parkinsonism model rats (Li et al., 2003). In order to elucidate if the anti-inflammatory activity is involved in the neuroprotective action, we investigated the effect of T_{10} on LPS-induced damage to dopaminergic neurons in primary embryonic midbrain cultures. It was found that T_{10} concentration-dependently attenuated the LPS-induced decrease in [^{3}H]dopamine uptake and loss of TH-ir neurons in vitro (Li et al., 2003), and the neuroprotective action might result from the inhibition of LPS-induced microglial activation and the release of cytotoxic compounds (Zhou et al., 2003). In the current in vivo study, we found for the first time that treatment with T_{10} for 24 days markedly prevented dopaminergic neuron degeneration in the SNpc and DA depletion in striatum found after LPS injection, and the underlying mechanism may be involved in its inhibitory effect on microglial activation induced by LPS intranigral injection. In agreement with our result, other immunosuppressive and anti-inflammatory drugs were also found to have neuroprotective effect on dopaminergic neurons. In MPTP-intoxicated mice, administration of the nonsteroidal anti-inflammatory drug aspirin has been shown to decrease the rate of dopamine depletion (Aubin et al., 1998). Similarly, administration of iNOS inhibitors, peroxisome proliferator activated receptors-gamma (PPARγ) inhibitors and cyclooxygenase 2 (COX-2) inhibitors also has been shown to confer neuroprotection against MPTP- and 6-OHDA-induced dopaminergic cell death in rodents (Barcia and Herrero Ezquerro, 2004; Barcia et al., 2003; Thomas and Le, 2004; Tomas-Camardiel et al., 2004).

The neuroinflammatory reaction is not specific to PD, as it has been conclusively observed in many other neurodegenerative diseases including Alzheimer's disease and multiple sclerosis. Neuroinflammatory processes possibly participate in the propagation of the neurodegenerative process in these diseases. Drugs targeting specific neuroinflammatory-associated deleterious mechanisms may prove effective in slowing or even halting the progressive neurodegeneration.

Conclusions

The present study demonstrated in vivo that microglial activation induced by intranigral injection of LPS had a degenerative effect on the dopaminergic neurons and T_{10}, a traditional Chinese herb with anti-inflammatory and immunosuppressive properties, can improve the survival of these injured neurons by inhibiting microglial activation and the release of proinflammatory cytokines, such as TNF-α and IL-1β. The previous study in our laboratory showed that T_{10} protected dopaminergic neurons from the lesion induced by the activation of the microglia challenged with LPS treatment in vitro. Taken together with our previous result, this study highly suggests the effectiveness of triptolide in protecting dopaminergic neurons against inflammatory challenge.

Acknowledgments

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