Triptolide inhibits TNF-α, IL-1β and NO production in primary microglial cultures

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Microglia are believed to participate in the mediation of neurodegeneration through producing a variety of cytotoxic factors upon activation. Pharmacological intervention in microglial activation may therefore exert a neuroprotective effect. In exploring pharmacological agents that can affect microglial activation, we found in this study that triptolide possesses a powerful inhibitory influence over microglia. Pretreatment with triptolide was able to dose-dependently reduce the lipopolysaccharide (LPS)-induced nitrite accumulation and tumor necrosis factor-α and interleukin-1β release from LPS-activated microglia as revealed by Griess reaction and ELISA, respectively. Triptolide reduced LPS-stimulated mRNA expression of all three inflammatory factors. The results obtained from this study demonstrate that triptolide can inhibit inflammatory responses of microglia to inflammatory stimulation via a mechanism involving the inhibition of the synthesis and release of inflammatory factors. *NeuroReport* 14:1091–1095 © 2003 Lippincott Williams & Wilkins.

Key words: Inflammation; Microglia; Neuroprotection; Nitric oxide; Proinflammatory cytokines; Triptolide; *Tripterygium wilfordii* Hook F.

INTRODUCTION

Inflammation in the central nervous system is closely associated with the pathogenesis of various neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, multiple sclerosis and AIDS dementia complex [1]. The hallmark of brain inflammation is the activation of microglia [2]. Upon activation, microglia can produce a variety of cytotoxic mediators, including nitric oxide (NO), proinflammatory cytokines, excitatory amino acid, reactive oxygen intermediates, and arachidonic acid and derivatives [3].

Due to the mediating role of microglia, a great deal of effort has been made to develop pharmacological treatment of neurodegenerative diseases by targeting microglia and associative inflammatory factors [4]. In our recent exploration of 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 [5]. Triptolide (known as PG490) has been identified as the major component responsible for the immunosuppressive and anti-inflammatory effects of TWHF [6]. Accumulating data have demonstrated a strong anti-inflammatory action of PG490 on multiple tissues. However, mechanisms underlying its anti-inflammatory action are poorly understood, especially its anti-inflammatory potential via a mechanism involving microglia.

This study was therefore designed to evaluate effects of PG490 on microglia, particularly their production of several key inflammatory factors. In a primary microglia-enriched culture system that we developed previously from neonatal rats, effects of PG490 on LPS-stimulated NO, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) release were tested. Putative effects of PG490 on inducible nitric oxide synthase (iNOS), TNF-α and IL-1β mRNA expression were then detected to define the PG490-sensitive regulation of these factors at the transcription level.

MATERIALS AND METHODS

Drugs and antibodies: PG490 was generously provided by Dr Jia-Run Zheng (Institute of Dermatology, Chinese Academy of Medical Sciences). The white crystal drug has a melting point of 226–240°C, and is 98% pure by reverse-phase high-pressure liquid chromatography evaluation. The mouse monoclonal antibody against the CR3 complement receptor (OX-42) was purchased from CHEMICON International, Inc. (Temecula, CA).

Primary microglia-enriched cultures: Primary microglia were isolated and purified from whole brains of neonatal 1-day-old male Sprague–Dawley rats according to procedures described previously [7]. Briefly, once animals were deeply anesthetized, their brains were dissected. The

experimental procedures were approved by the Committee on Animal Care and Usage of Peking University Health Science Center, and all efforts were made to minimize animal suffering. The meninges were removed carefully and the tissues were minced and digested with 0.125% trypsin for 25 min. After mechanical dissociation, the cells were resuspended in a serum-containing medium: DMEM/F12 (1:1; Gibco Life Technologies, Rockville, MD), 25 U/ml penicillin, 25 μg/ml streptomycin and 10% fetal calf serum (Gibco Life Technologies, Rockville, MD). The cells were seeded in 75 cm^2 flasks at a density of 2.5×10^7 cells/flask. Two weeks later, the flasks were shaken at 200 r.p.m. for 4 h. The floating cells were collected and replanted. Three hours later, when most microglia were adhering to the substrates, the plate was tapped gently to remove loosely adhering oligodendrocytes. The purity of the microglial-enriched culture was confirmed by OX-42 immunocytochemistry, which showed that $> 95\%$ of cells were immunoreactive to the microglia-specific marker OX-42. The remaining monolayer of purified microglia was cultured in serum-containing medium/N2 (1:1) for 24 h before the medium was changed to N2 (Sigma Chemical Co., St. Louis, MO) for further experimental treatment.

Nitrite assay: The accumulation of nitrite in the culture supernatant, an indicator of the production of NO, was determined with a calorimetric assay using Griess reagent from Fluka Chemie AG (Switzerland) (0.1%. N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 5% H_3PO_4) as described previously [8]. Briefly, primary microglial cells were seeded on 96-well plate at a density of 2×10^4/well. After treatment with LPS and/or PG490, 50 μl supernatant was transferred to a new 96-well plate and an equal volume of the Griess reagent was added. The absorbance at 570 nm−655 nm was determined with a microplate reader (BIO-RAD Laboratories, CA). The concentrations of nitrite in the samples were determined from a sodium nitrite standard curve. For statistical analysis, a single treatment was performed on four individual wells.

TNF-α and IL-1β ELISA: Primary microglial cells were seeded on 96-well plate at a density of 2×10^4/well. After treatment with LPS and/or PG490, 100 μl supernatant was collected and stored at 4°C. If not used immediately, the supernatant was stored at −80°C until assays for TNF-α and IL-1β were performed. TNF-α and IL-1β levels were detected by rat TNF-α and IL-1β ELISA kits (Pierce Biotechonology, Inc., Rockford IL), 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. For statistical analysis a single treatment was performed on four individual wells.

RT-PCR analysis of iNOS, IL-1β and TNF-α gene expression: A semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay was used to determine the mRNA levels of iNOS, IL-1β or TNF-α in relation to GAPDH message as described previously [9]. Briefly, total RNA was extracted from primary microglia by Trizol reagent (Invitrogen Corporation, Carlsbad, CA). Approximately 2 μg total RNA was used for cDNA synthesis by reverse transcription with 200 U M-MLV reverse transcriptase (Invitrogen Corporation, Carlsbad, CA) in an RT buffer in the presence of 0.5 mM dNTPs, 30 U RNase inhibitor, and 0.5 μg oligodT as primers. The thermal cycler was programmed for 60 min at 42°C, 5 min at 95°C. A 4 μl aliquot of cDNA synthesized in the RT reaction was used for PCR amplification in the presence of 1 U Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA) in Taq buffer, 0.2 mM each of dNTPs and 1 μM of each primer. The iNOS was amplified for 21 cycles using a three-step program (30 s at 94°C, 30 s at 60°C, 1 min at 72°C). TNF-α and IL-1β were amplified for 27 cycles using a three-step program (30 s at 94°C, 30 s at 60°C, 1 min at 72°C for TNF-α and 30 s at 94°C, 1 min at 60°C, 1 min at 68°C for IL-1β). After amplification, the products were separated on an agarose gel in the presence of ethidium bromide and visualized under u.v. light. The following sequences of the primers were adopted from previously published reports [9,10]. iNOS (upstream), 5′-CGTGTGCCTGCTGCCTTCC-TGCTGT-3′; iNOS (downstream), 5′-GTAATCCTCAA-CCTGCTCCTCACTC-3′. TNF-α (upstream), 5′-CACGCTC-TTCTGTCTACTGA-3′; TNF-α (downstream), 5′-GGAC-TCCGTGATGTCTAAGT-3′. IL-1β (upstream), 5′-AAGC-TCTCCACCTCAATGGACAG-3′; IL-1β (downstream), 5′-CTCAAACTCCACTTTGCTCTTGA-3′. GAPDH (upstream), 5′-TCCCTCAAGATTGTCAGCAA-3′; GAPDH (downstream), AGATCCACAACGGATACATT-3′.

Statistical analysis: Data are expressed as means ± s.e. m. Statistical significance was assessed by one-way ANOVA followed by Newman–Keuls *post hoc* test of difference between groups. A value of $p < 0.05$ was considered statistically significant.

RESULTS

PG490 reduced LPS-induced formation of nitrite: Effects of PG490 on the LPS-induced nitrite formation were evaluated. Microglial cultures were incubated with PG490 for 30 min prior to and subsequently during 24 h treatments with LPS. Culture supernatants were collected for determining the levels of nitrite. As shown in Fig. 1, PG490 at concentrations of 10^{-10} and 10^{-9} M had no significant effect on LPS-induced accumulation of nitrite. PG490 at concentrations up to 10^{-8} and 10^{-7} M significantly inhibited the accumulation of nitrite induced by 0.25 μg/ml LPS by 54% and 91%, respectively. At concentrations of 10^{-8} and 10^{-7} M, PG490 alone did not affect the viability of primary microglial cells revealed by the method of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion (data not shown).

PG490 inhibited LPS-induced formation and release of TNF-α and IL-1β: To examine the effect of PG490 on LPS-induced generation and release of proinflammatory cytokines, primary microglial cells were pretreated with PG490 for 30 min followed by co-treatment with 0.25 μg/ml of LPS for 24 h. Supernatants were then taken for the measurement of levels of proinflammatory cytokines TNF-α and IL-1β. From Fig. 2a, the increased TNF-α level induced by LPS was moderately reduced by PG490 at a low-to-middle dose range (10^{-10}–10^{-8} M). A nearly complete blockade of the

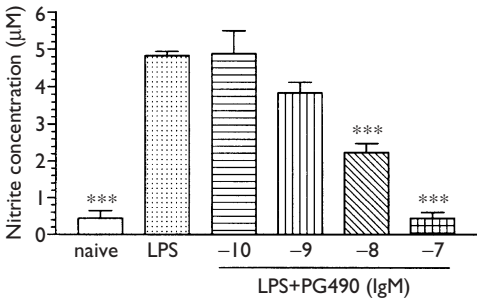

Fig. 1. Effect of PG490 on LPS-stimulated production of NO. Primary microglia were pretreated with PG490 at different concentrations for 30 min followed by co-treatment with 0.25 μg/ml LPS for 24 h. Culture supernatants were collected and assayed for nitrite with the Griess reagent. The results are the means ± s.e.m. of three experiments performed in quadruplicate. ***$p < 0.001$ compared with the LPS-treated cultures.

TNF-α induction was achieved after PG490 treatment at a high dose (10^{-7} M). PG490 also reduced IL-1β induction by LPS (Fig. 2b). However, consistent and significant reduction was seen only at the two higher concentrations (10^{-8} and 10^{-7} M).

PG490 inhibited LPS-stimulated iNOS, TNF-α and IL-1β gene expression: To determine the mechanism by which PG490 inhibited the production of NO, TNF-α and IL-1β, we examined the levels of iNOS, TNF-α and IL-1β mRNA using RT-PCR. Microglial cells were pretreated with PG490 for 30 min followed by co-treatment with 0.25 μg/ml LPS for 6 h. The cells were then collected for RT-PCR detection of mRNAs of interest. Since levels of GAPDH were not changed by any drug treatments (Fig. 3), levels of the target mRNAs were normalized against GAPDH mRNA. While a low, but detectable, level of IL-1β mRNA was present in untreated primary microglia, constitutive iNOS and TNF-α mRNAs were not detectable in untreated microglia (Fig. 3). LPS treatment (0.25 μg/ml; 6 h) significantly elevated the levels of iNOS (Fig. 3a), TNF-α (Fig. 3b) and IL-1β (Fig. 3c). PG490 (10^{-7} M) reduced LPS-induced increases in all three mRNAs (Fig. 3).

DISCUSSION

Using primary microglia-enriched culture, this study has demonstrated for the first time that PG490 significantly inhibits LPS-induced activation of microglia and the resultant increase in the steady state levels of NO, TNF-α and IL-1β mRNA.

Microglia are the main immune cells in the CNS. The traditional view favors a mere scavenger role of microglia so that microglial reaction is considered to be a secondary event to neuronal damage. However, recent studies demonstrate a more active contribution of microglia to the development of progressive neurodegenerative disease [11]. Le *et al.* have demonstrated that LPS-induced microglial activation leads to injury of neurons *in vitro* [11]. *In vivo* experiments also demonstrated that injection of LPS into the substantia nigra induced microglial activation and damage in nigral dopaminergic neurons [12]. In brief, both *in vitro*

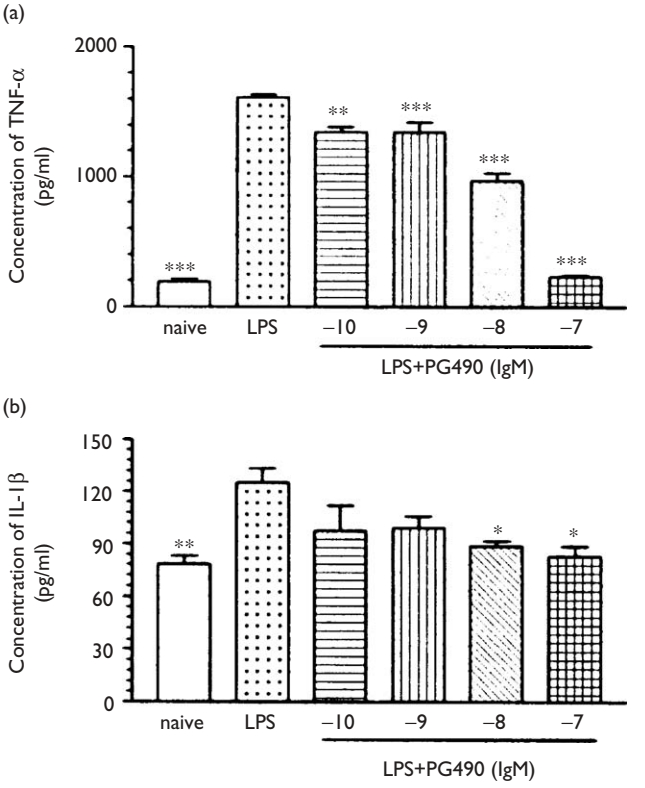

Fig. 2. Effect of PG490 on LPS-induced release of TNF-α and IL-1β. Primary microglia were pretreated with PG490 at different concentrations for 30 min followed by co-treatment with 0.25 μg/ml LPS for 24 h. Culture supernatants were collected and assayed for TNF-α (**a**) and IL-1β (**b**) with commercial ELISA kits. The results are the means ± s.e.m. of three experiments performed in quadruplicate. *$p < 0.05$ compared with the LPS-treated cultures. **$p < 0.01$ compared with the LPS-treated cultures. ***$p < 0.001$ compared with the LPS-treated cultures.

and *in vivo* experiments have shown that activated microglia may have detrimental effects on neurons.

Central to understanding the detrimental effects of microglia is the fact that, upon activation, they release a wide spectrum of proinflammatory and cytotoxic factors, including NO, TNF-α and IL-1β [9]. It is generally thought that NO released from activated microglia may mediate neuronal toxicity through mitochondrial dysfunction [13]. Moreover, NO can interact with O_2^- to form the highly toxic free radicals peroxinitrite ($ONOO^-$), which can attack protein, DNA and lipid directly [14]. Therefore, the inhibition of production of NO can protect neurons from damage [15]. TNF-α is one of the most important proinflammatory cytokines in the CNS: it can up-regulate expression of MHC class I in neurons, which makes them susceptible targets for class I-restricted cytotoxic T cells [16]. TNF-α also interferes with the electrophysiology of neurons: *in vitro* studies have shown that TNF-α affects ion currents, intracellular Ca^{2+} homeostasis and membrane potentials and suppresses long-term potentiation, thus contributing to dysfunction in inflammation [17]. Another proinflammatory cytokine, IL-1β, is known to be the major microglial signal that promotes the cascade of glial cell reactions [18]. The pathologically stimulated release of IL-1β from microglial cells triggers secondary activation of astrocytes, which are

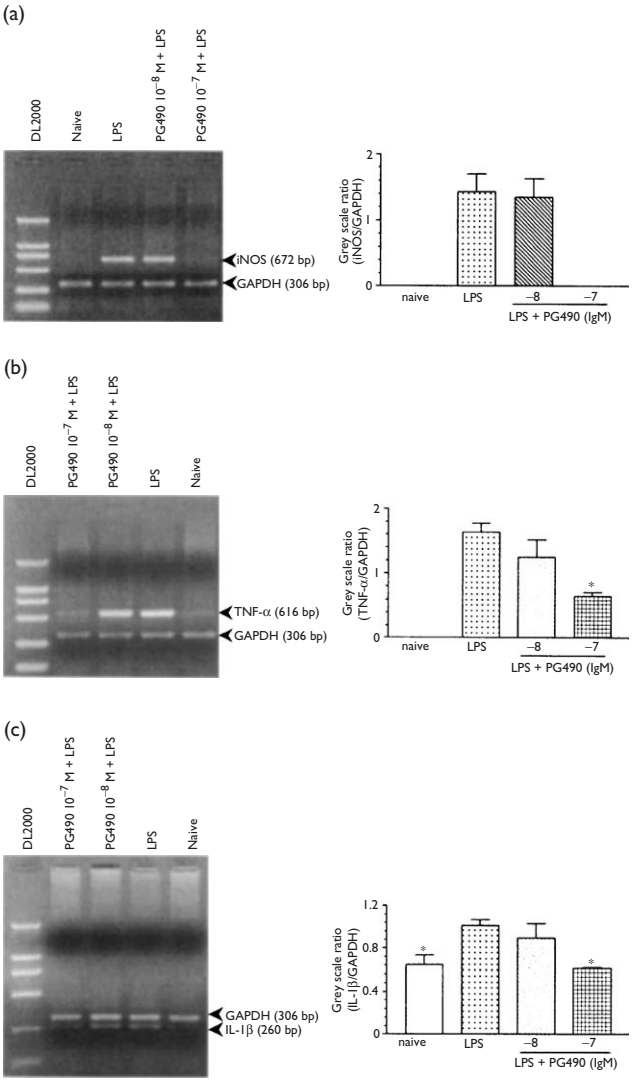

Fig. 3. Effect of PG490 on LPS-induced iNOS, TNF-α, and IL-1β transcription. Primary microglia were pretreated with 10^{-8} M or 10^{-7} M PG490 for 30 min followed by co-treatment with 0.25 μg/ml LPS for 6 h. Transcription of iNOS, TNF-α, and IL-1β was detected by semiquantitative RT-PCR. After amplification, the products were separated on an agarose gel in the presence of ethidium bromide and visualized under u.v. light (left side). The grey ratio of iNOS (**a**), TNF-α (**b**), and IL-1β (**c**) to GAPDH from three independent experiments was calculated (right side). $*p < 0.05$ compared with the LPS-treated cultures.

forced to proliferate and to give up their differentiated state. As a consequence, physiologically required astrocyte functions, such as uptake of glutamate and K^+ from the extracellular space and the release of neurotrophic factors [17], may be impaired. Therefore, a pharmacological inhibition of microglial TNF-α and IL-1β release can be expected to contribute to neuroprotection [19]. Taken together, accumulating data have demonstrated that excessive production and accumulation of inflammatory factors contribute to neurodegeneration and that prevention of microglial activation serves to reduce neuronal injury.

Due to the mediating role of microglia, a great deal of effort has been made to develop neuroprotective strategies by targeting microglia and associative inflammatory factors [20]. In this study we have demonstrated that PG490 inhibited LPS-induced microglial activation, their production of NO, TNF-α and IL-1β.

PG490 is an oxygenated diterpene derived from a traditional Chinese herb that has been used as an immunosuppressant in China for the treatment of rheumatoid arthritis [21], but the mechanism of this therapeutic effect has not been completely delineated. Recent studies have shown that PG490 inhibits T-cell activation and early cytokines gene transcription in T-cell and epithelial cells at the purine-box/ARRE/NF-AT and NF-κB target sequence after specific DNA binding [22,23]. In the microglia, NF-κB is also the key transcription factor that governs the expression of genes encoding proinflammatory cytokines and enzymes related to the inflammatory process, including TNF-α, IL-1β and iNOS. The inhibitory effect of PG490 on the steady state levels of mRNAs encoding the factors assayed in the present study may arise from similar mechanism.

The results obtained in this study are a significant extension of our previous report in which we showed that tripchlorolide, a structural analogue of PG490, protected dopaminergic neurons from degeneration induced by 1-methyl-4-phenylpyridinium ion (MPP^+) and axotomy [24]. While this previous study suggested neuroprotective activity of tripchlorolide, the underlying mechanism was not determined. In this study, we demonstrated that PG490 had inhibitory effect over microglial activation, which may be one of the main mechanisms of the neuroprotective activity. In view of the fact that TWHF has been used clinically for the treatment of rheumatoid arthritis for a long time, it is quite conceivable that further research along this line may pave a new path for the treatment of neurodegenerative diseases.

In summary, inflammatory factors produced by activated microglia contribute to neurodegeneration and prevention of microglia activation serves to reduce neuronal injury. In exploring pharmacological agents that can affect microglial activation, we found in this study that PG490 can inhibit the activation of primary microglia, their production of NO, TNF-α, and IL-1β at the transcription level. In view of the fact that PG490 inhibits T-cell activation and cytokines gene transcription by inhibiting the activity of NF-AT and NF-κB, and NF-κB is also the key transcription factor in microglia, the inhibitory effect of PG490 on the steady state levels of mRNAs encoding these inflammatory factors may arise from similar mechanism.

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