ORIGINAL ARTICLE

The aqueous extract from *Toona sinensis* leaves inhibits microglia-mediated neuroinflammation

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Abstract   The leaves of *Toona sinensis*, a well-known traditional oriental medicine, have been prescribed for the treatment of enteritis and infection. Recently, aqueous extracts of *Toona sinensis* leaves (TSL-1) have demonstrated many biological effects both *in vitro* and *in vivo*. In the central nervous system, microglial activation and their proinflammatory responses are considered an important therapeutic strategy for neuroinflammatory disorders such as cerebral ischemia, Alzheimer's disease, and Parkinson's disease. The present study attempted to validate the effect of TSL-1 on microglia-mediated neuroinflammation stimulated by lipopolysaccharide (LPS). As inflammatory parameters, the production of nitric oxide (NO), inducible NO synthase, and tumor necrosis factor-α were evaluated. Our results demonstrate that TSL-1 suppresses LPS-induced NO production, tumor necrosis factor-α secretion, and inducible NO synthase protein expression in a concentration-dependent manner, without causing cytotoxicity. In addition, the inhibitory effects of TSL-1 in LPS-stimulated BV-2 microglia were extended to post-treatment suggesting the therapeutic potential of TSL-1. Therefore, this work provides the future evaluation of the role of TSL-1 in the treatment of

neurodegenerative diseases by inhibition of inflammatory mediator production in activated microglia.

Introduction

Inflammation is known to play a key role in the progressive damage process in a number of neurodegenerative disorders including Alzheimer's disease (AD) [1,2], Parkinson's disease (PD) [3], multiple sclerosis [4,5], and stroke [6,7]. Microglia act as the major immune cells in the central nervous system. In response to brain injury or during neurodegenerative processes, microglia are activated by secreting growth factors, proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and nitric oxide (NO), and reactive oxygen species [3,8–11]. Although microglial activation is necessary and important for host defense, over-activation of microglia is neurotoxic. Studies have shown that microglia activated after ischemic stroke will produce cytokines to trigger neuronal death in response to ischemic injury [6,12]. They also found that the inhibition of inflammation would prevent the progressive brain loss following a stroke. Thus, to develop the agents that reduce microglial activation and their proinflammatory responses is considered an important therapeutic strategy for neuroinflammatory disorders such as cerebral ischemia, AD, and PD.

Toona sinensis (TS), a well-known Chinese herb, is widely available in Asia. All parts of TS, including its root, bark, petioles, leaves, fruits, and seeds, have been used for medicinal purposes [13–15]. The leaves of TS are a popular vegetable amongst vegetarians in Taiwan. It also serves as an ingredient in some Chinese and Malaysian recipes. In the field of traditional Chinese medicine, the leaves of TS have been used for treating enteritis, dysentery, diabetes, infection, and itch, with no irreversible side effects observed after treatment [16]. Recent studies have also revealed that the aqueous extracts of TS leaves (TSL-1) have a variety of biological functions, including: (1) antioxidant activities [17,18]; (2) the lowering of blood sugar levels via mediating adipose glucose transporter [19,20]; (3) the alleviation of liver fibrosis via reducing tumor growth factor-β1 and collagen [21]; (4) the inhibition of coronavirus replication in severe acute respiratory syndrome [22]; (5) the decrease of steroidogenesis in mouse Leydig cells [23]; and (6) the inhibition of vascular endothelial growth factor (VEGF)-induced angiogenesis in vascular endothelial cells [24]. In addition, TSL-1 has antiproliferative properties in human lung cancer cells [25–28], oral squamous carcinoma cells [29], and human premyelocytic leukemia cells [30] *in vitro*. Furthermore, there was no acute lethal effect even at a maximal oral tested dose of 5000 mg/kg of body weight in mice [31,32]. Interestingly, one of these findings demonstrated that daily dietary supplement of TSL-1 in senescence-accelerated mice (an AD model) improved brain degeneration caused by the incidence of β amyloid plaques [17]. This points to the possibility that TSL-1 may pass through the blood–brain barrier to affect the central nervous system. Recent studies have also found that the appearance of amyloid plaques in the brain coincides with a dramatic phenotypic activation of the surrounding microglia, which release proinflammatory cytokines and neurotoxic substances, for disease progression [33,34]. Liao et al.'s findings [17] indicated that TSL-1 supplement in aged mice had a potential effect on neuroinflammation due to the recruitment of activated microglia in amyloid plaques. Therefore, this study aimed to examine whether TSL-1 would modulated neuroinflammation-associated diseases such as AD through microglia. At the same time, because the systemic administration of lipopolysaccharide (LPS, a heat-stable bacterial cell wall component) in mouse brain, causing amyloid protein accumulation and neuroinflammation, was being used to study the underlying mechanisms of AD [35–37], we tested the potential anti-inflammation effect of TSL-1 in the *in vitro* model of LPS-induced microglial activation system.

Materials and methods

Plant materials and preparation of TSL-1

TS leaves were obtained in Tuku (Yunlin County, Taiwan). The leaves were picked and washed with water as described by Chang et al. in 2002 [25]. Reverse osmosis water was added to TS leaves at a proportion of 4 L of reverse osmosis water to 1 kg of leaves. The mixture was boiled for 30 minutes, after which the leaves were removed and the remaining liquid concentrated over low heat and filtered with a sieve (70 meshes). The filtered concentrate was lyophilized with a Virtis apparatus (Gardiner, NY, USA) to obtain a crude extract. The crude extracts were centrifuged at 1400g for 12 minutes, and the supernatant-labeled TSL-1 (an advanced bioactive fraction of TS) was used for this study. The extracts were then concentrated in a vacuum, freeze-dried to form a powder, and stored at −20°C for subsequent analysis. Various doses of TSL-1, from 5 μg/mL to 50 μg/mL, were used in this study.

BV-2 microglial cell culture

A murine cell line (BV-2) was generated by infecting primary microglial cell cultures with a v-raf/v-myc oncogene carrying retrovirus (J2), with most of the morphological, phenotypical, and functional properties described for freshly isolated microglial cells retained [38]. In the present study, BV-2 microglial cell line was a gift from Professor Hong, Jau-Shyong (Research Triangle Park, NIEH; NIH, Bethesda, MA, USA). Cells (1 × 10^5 cells/mL) were cultured

in Dulbecco's modification of Eagle's medium (DMEM; GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, ThermoFisher Scientific, New Zealand), 100 U/mL penicillin—streptomycin (GIBCO), and 4 mM L-glutamine (GIBCO) and were maintained in a 5% carbon dioxide incubator. In all experiments except the post-treatment scheme, cells were treated with TSL-1 (5 μg/mL, 10 μg/mL, or 50 μg/mL) 30 minutes prior to the addition of lipopolysaccharide (LPS, 1 μg/mL, *Escherichia coli*, Serotype 055:B5; Sigma-Aldrich, St Louis, MO, USA) in DMEM with 2% FBS. Passages 3—8 of the BV-2 cell lines were used in this study.

Cell viability assay

BV-2 microglial cells (1×10^5 cells/mL, 24-well plate) were allowed to adhere and grow overnight. Cells were then incubated in 2% FBS-containing medium with different concentrations of TSL-1 for 6 hours and 24 hours. After incubation, 2 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) reagent replaced the medium and was incubated in a 5% carbon dioxide incubator at 37°C for an additional 4 hours. The BV-2 cells were then harvested in 50 μL dimethyl sulfoxide and transferred to 96-well plates. The absorbance was measured at 540 nm using a microplate reader (Beckman Coulter Inc., Brea, CA, USA).

Measurement of TNF-α production by enzyme-linked immunosorbent assay

BV-2 microglial cells were pretreated with medium or TSL-1 (50 μg/mL) for 30 minutes prior to incubation with LPS (1 μg/mL) in 24-well plate. At different time points (6 hours and 24 hours after treatment with LPS), the supernatants were collected for TNF-α measurements by enzyme-linked immunosorbent assay kits (Endogen mouse/rat TNF-α ELISA kit; Thermo Fisher Scientific, Rockford, IL, USA). Each sample was tested in duplicate.

NO analysis

NO was evaluated by measuring the amount of nitrite in the cell culture supernatant, using Griess reagent (0.1% naphthylethylenediamine, 1% sulfanilamide in 2.5% H_3PO_4). BV-2 microglial cells were grown on 24-well plates and pretreated with medium or TSL-1 (5 μg/mL, 10 μg/mL, or 50 μg/mL) for 30 minutes prior to incubation with LPS (1 μg/mL). For the post-treatment study, TSL-1 (50 μg/mL) was added during, or 1 hour, 2 hours, 4 hours, or 6 hours after, LPS (1 μg/mL) treatment in BV-2 microglial cell cultures. The supernatant were collected 24 hours after LPS treatment. The production of NO was determined basing on the Griess reaction. In short, 50 μL of culture supernatant was allowed to react with an equal volume of Griess reagent in 96-well plates for 10 minutes at room temperature in the dark. The absorbance at 550 nm was determined using a microplate reader (MRX ELISA reader; Dynex, Chantilly, VA, USA). A standard nitrite curve was generated in the same fashion using $NaNO_2$.

Western blot analysis

BV-2 microglial cells were grown on 6-well plates and pretreated with medium or TSL-1 (5 μg/mL, 10 μg/mL, or 50 μg/mL) for 30 minutes prior to incubation with LPS (1 μg/mL). For the post-treatment study, TSL-1 (50 μg/mL) was added during, or 1 hour, 2 hours, 4 hours, or 6 hours after, LPS (1 μg/mL) treatment in BV-2 microglial cell cultures. After treatment, cell lysates were washed twice with phosphate-buffered saline and harvested in Laemmli sodium dodecyl sulfate sample buffer. The protein concentration in the supernatant was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of whole cell lysates were separated in 10% sodium dodecyl sulfate—polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were first incubated with 5% nonfat milk in PBS for 1 hour at room temperature to reduce nonspecific binding. The membranes were washed with PBS containing 0.1% Tween-20, and then incubated for 1 hour at room temperature with the indicated antibodies including inducible NO synthase (*i*NOS; 1:1000; BD Biosciences, Franklin Lakes, NJ, USA), HO-1 (1:10,000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and β-actin (1:20,000, Sigma-Aldrich). This was followed by the addition of horseradish peroxidase-conjugated secondary antibody. After the final wash, membranes were probed using enhanced chemiluminescence (Amersham Pharmacia Biotech) and autoradiographed. The optical density of the bands (integrated area, arbitrary units) was measured by an Imaging Densitometer (Bio-1D V.97; Vilber Lourmat, Torcy, France).

Statistical analysis

Data are expressed as mean ± standard deviation for the separate experiments. The differences among treatment groups were determined by analysis of varience (ANOVA) with *post hoc* compared Dunnett's test with $p < 0.05$ as the criterion of significance. The statistical analysis was computed by SAS 9.20 (SAS Institute, Inc., Chicago, IL, USA).

Results

Effect of TSL-1 on the cell viability of BV-2 microglial cells

To test whether TSL-1 treatment would affect the cell viability of BV-2 microglial cells, cells were incubated in different concentrations of TSL-1 for 6 hours and 24 hours. In the normal condition without TSL-1 treatment, there was an increase in BV-2 microglial cell viability at 24 hours' incubation when compared to 6 hours of incubation (Fig. 1). Within our tested concentration range of TSL-1 (5—50 μg/mL), the cell viability was comparable to those corresponding controls at 6 hours' and 24 hours' incubation (Fig. 1). At the same time, there was no cell death found (data not shown). The TSL-1 treatment alone did not change the cell viability of BV-2 microglial cells.
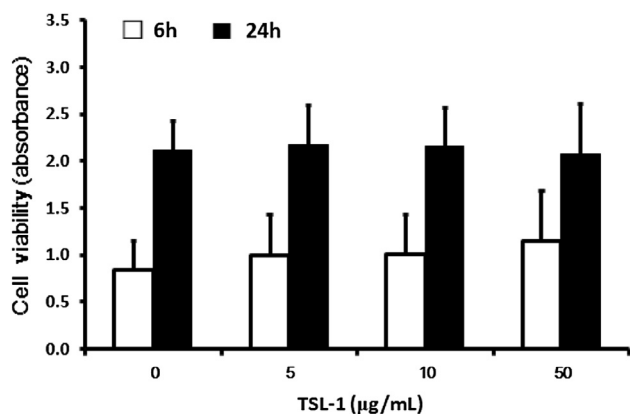
Figure 1. Effect of aqueous extract of *Toona sinensis* leaves (TSL-1) on cell viability of BV-2 microglial cells. BV-2 microglial cells were incubated with various concentrations of TSL-1 for 6 hours and 24 hours. MTT assay was performed to detect viability of the cells and the results were expressed as the absorbance at 570 nm. Six independent experiments were performed at each time and dose points. Data are expressed as mean ± standard deviation.

Effect of TSL-1 on BV-2 microglial cell morphology after LPS stimulation

Traditionally, the microglial cells were classified into two primary phenotypic states *in vivo*: "quiescent" or "activated". The transformation of "quiescent" microglia with ramified morphology to the "activated" phenotype with round or amoeboid shape was associated with inflammation and disease [39]. LPS, as a potent activator of microglia, will stimulate microglia to become activated and to undergo a series of morphologic and phenotypic changes [40].

In this study, BV-2 microglial cell morphology was observed by phase-contrast microscope. In the treatment of LPS alone for 24 hours, BV-2 cell morphology transformed from a predominantly rod cell morphology to a round or oval shape (Fig. 2). Clustering of BV-2 cells was usually observed in the LPS-alone plates. There were no compatible morphologic changes between the control and TSL-1 alone plates. In the group of pretreatment with TSL-1 (50 μg/mL) for 30 minutes then incubated with LPS for an additional 24 hours, although some cell debris was found, the BV-2 cell morphology showed the same shapes as the controls and TSL-1 alone ones.

TSL-1 inhibited LPS-induced TNF-α production in BV-2 microglial cells

As demonstrated in Fig. 3, treatment of BV-2 cells with LPS (1 μg/mL) caused a substantial increase in the production of TNF-α, dependent on time. Pretreatment with TSL-1 (50 μg/mL) prior to incubation with LPS resulted in a significant inhibition of the LPS-induced TNF-α production in both the 6 hours and 24 hours treatment groups ($p < 0.001$
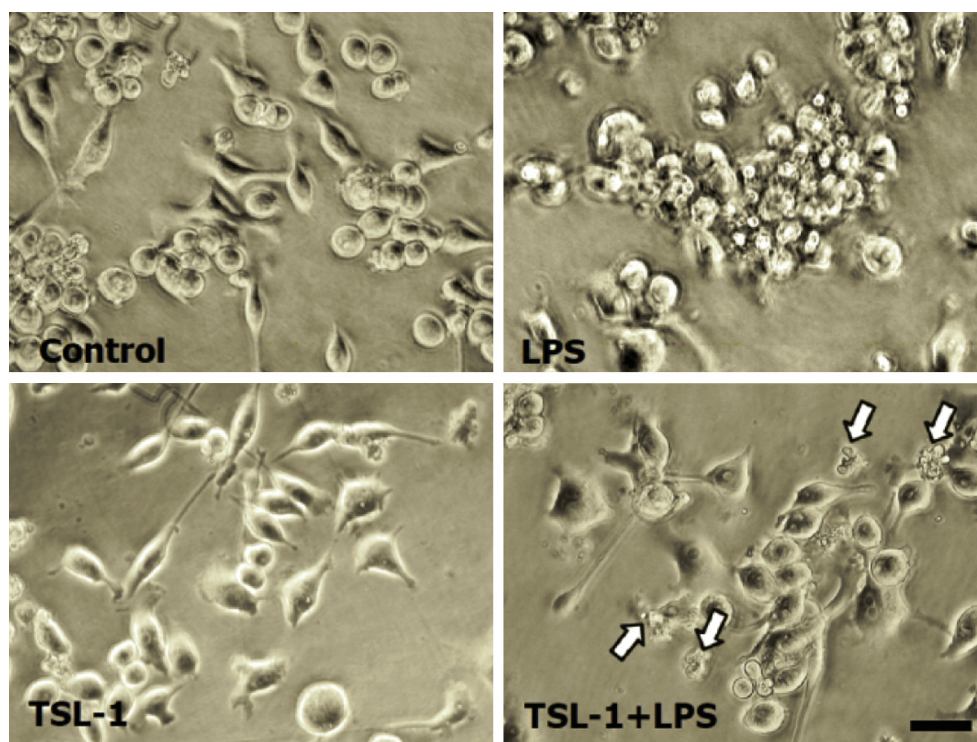

Figure 2. Effect of aqueous extract of *Toona sinensis* leaves (TSL-1) on BV-2 microglial cell morphology. BV-2 microglial cells were pretreated with medium or TSL-1 (50 μg/mL) for 30 minutes prior to stimulation with lipopolysaccharide (LPS; 1 μg/mL) for an additional 24 hours. The photomicrographs were taken directly from culture plates by converted light microscopy with phase contrast. Some cell fragments (arrows) were found in the group pretreated with TSL-1 for 30 minutes then incubated with LPS for an additional 24 hours (TSL-1 + LPS). Control group: cell incubated in the 2% FBS medium for 24 hours. TSL-1 group: cells incubated in 2% FBS medium including 50 μg/mL of TSL-1 for 24 hours. Three independent experiments were performed. Scale bar = 20 μm.
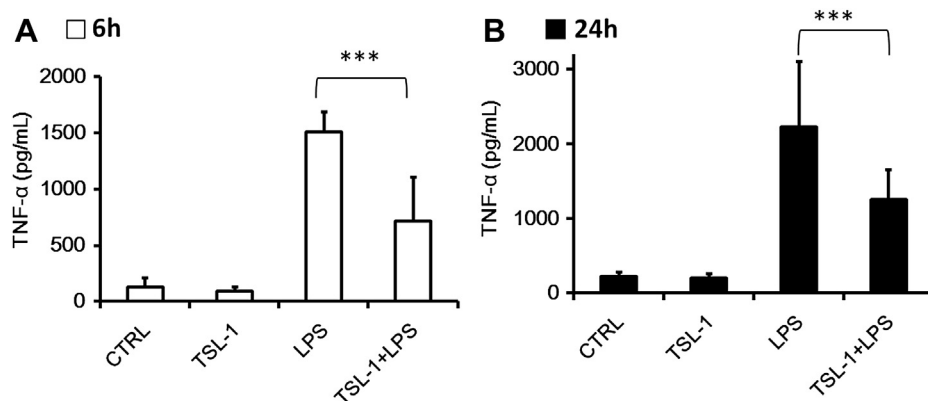
Figure 3. Pretreatment with aqueous extract of *Toona sinensis* leaves (TSL-1) reduced the production of tumor necrosis factor-α in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. BV-2 microglial cells were pretreated with TSL-1 (50 μg/mL) for 30 minutes prior to incubation with LPS (1 μg/mL). At different time points (6 hours and 24 hours after treatment of LPS), the supernatants from BV-2 cell cultures were collected for tumor necrosis factor-α measurements by enzyme-linked immunosorbent assay (6 hours in A, 24 hours in B). Data are expressed as mean ± standard deviation from five independent experiments in triplicate. The significance of the differences among treatment groups were determined by ANOVA (***$p < 0.001$).

for both). There was no effect on the TNF-α production in BV-2 cells treated with TSL-1 alone for 6 hours and 24 hours.

Pretreatment with TSL-1 reduced the NO production and iNOS protein expression in LPS stimulated BV-2 microglial cells

Previous study has demonstrated that NO would be released from microglia following exposure to LPS [41]. In this series of experiments (Fig. 4A), treatment of BV-2 microglial cells with LPS (1 μg/mL) for 24 hours caused a robust increment of NO level (11.8 ± 2.44μM). Pretreatment with TSL-1 prior to incubation with LPS resulted in a concentration-dependent inhibition of the LPS-induced NO production in BV-2 cells (TSL-1 5 μg/mL + LPS: 7.7 ± 2.33, TSL-1 10 μg/mL + LPS: 7.5 ± 1.96, TSL-1 50 μg/mL + LPS: 3.8 ± 1.5, $n = 5$). Moreover, the LPS treatment markedly increased the protein level of iNOS in BV-2 microglial cell culture as with as little as 6 hours' incubation. This induction was drastically inhibited by pretreatment with TSL-1 in a time- and concentration-dependent manner (Fig. 4B,C).

Post-treatment effect of TSL-1 on NO production and iNOS protein expression in LPS-stimulated BV-2 microglial cells

In addition to pretreatment, we also evaluated the effects of post-treatment with TSL-1 on the LPS-induced microglial activation. TSL-1 (50 μg/mL) was added during, or 1 hour, 2 hours, 4 hours, or 6 hours after LPS (1 μg/mL) treatment in BV-2 cells. Supernatant and cell lysates were collected after 24 hours of LPS incubation for the detection of NO production. As shown in Fig. 5A, LPS treatment significantly increased NO release from BV-2 microglial cells. Post-treatment with TSL-1 up to 6 hours after LPS treatment attenuated LPS-induced release of NO in BV-2 microglial cells by 50% to 80%. A similar pattern was observed in the group of post-treatment with TSL-1 on the LPS-induced

iNOS (Fig. 5B) production. When the iNOS production at 24 hours after LPS treatment was determined, the addition of TSL-1 at 0 hours and 1 hour after LPS treatment still exhibited an inhibitory effect on LPS-induced iNOS production (0 hours: 47.5%; 1 hour: 50.18% of LPS alone). However, the addition of TSL-1 at 2 hours after LPS treatment showed no obvious inhibitory effect on iNOS production.

Discussion

This is the first report to demonstrate that TSL-1 markedly inhibited LPS-induced inflammatory responses in the murine microglial BV-2 cell line. NO production and iNOS expression were significantly inhibited by TSL-1 in a concentration-dependent manner in the microglial BV-2 cell line. This anti-inflammatory effect of TS was also evidenced by inhibiting TNF-α release. Moreover, the cell viability assay showed that treatment with TSL-1 alone did not have cytotoxic effects at concentrations of 5–50 μg/mL, whereas TSL-1 significantly inhibited those inflammatory factors stimulated by LPS. In this connection, we suggest that TSL-1 might have a potent antineuroinflammatory activity via the inhibition of LPS-stimulated production of TNF-α, NO, as well as iNOS protein in microglia.

In the present study, we also used microglia BV-2 cell to evaluate the potential therapeutic effect of TSL-1 after LPS treatment. The results indicated that even post-treatment with TSL-1 (50 μg/mL), later than LPS application, is effective in the reduction of NO release and iNOS protein level. Accumulating evidence indicates that iNOS is the most important contributor to NO production in the brain after inflammatory assault, compared with other isoforms of NOS, namely eNOS and nNOS [42,43]. Furthermore, a novel approach with NO-donating nonsteroidal anti-inflammatory drugs develops a safe profile that strongly reduces their untoward side effects without altering the anti-inflammatory effectiveness [44–47]. These findings
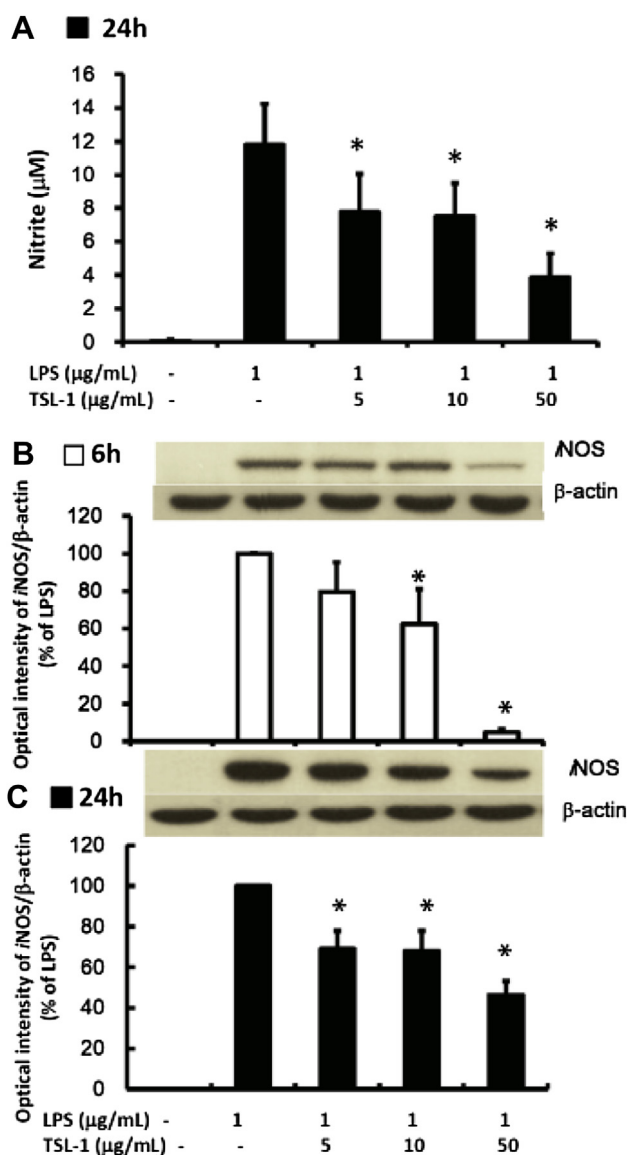
A ■ 24h

B □ 6h

C ■ 24h

Figure 4. Pretreatment with aqueous extract of *Toona sinensis* leaves (TSL-1) reduced the NO production and iNOS protein expression in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. Cells were pretreated with medium or TSL-1 (5 μg/mL, 10 μg/mL, and 50 μg/mL) for 30 minutes in the presence of LPS (1 μg/mL) for an additional 6 hours and 24 hours, respectively. (A) The supernatant for NO analysis was collected at 24 hours of LPS incubation. Nitrite levels in cultured media were determined by Griess assay and were reflected to NO levels. Sodium nitrite was used for preparation of the standard curve. Results were presented as means ± standard deviation from five independent experiments in quadruplicates. (B,C) For the analysis of iNOS activity, equal amounts of cell lysates in each experiment were collected at 6 hours and 24 hours of LPS incubation. The iNOS expressions were examined by immunoblotting. Data are expressed as a percentage of the values of LPS group. The bar graph shows the densitometric analysis for five blots from four independent experiments (mean ± standard deviation). Beta-actin was used as internal control.*$p < 0.05$ versus the LPS-treated alone group.
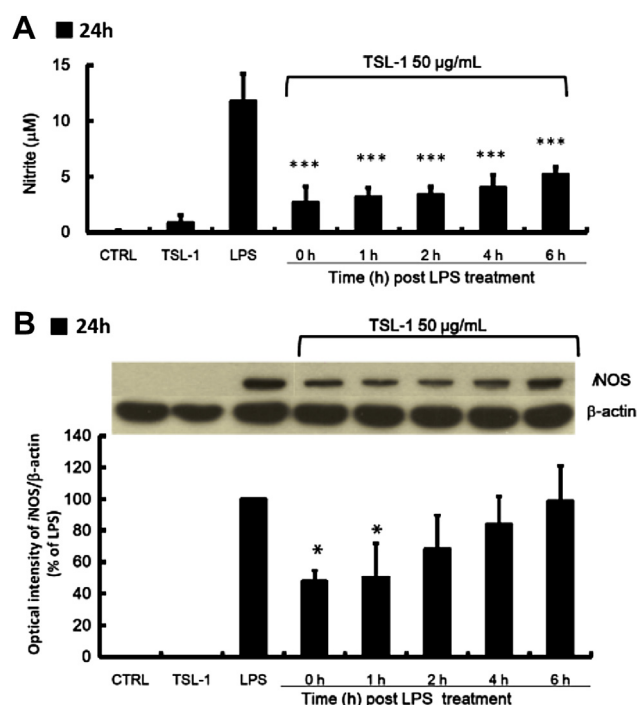
A ■ 24h

B ■ 24h

Figure 5. Effects of aqueous extracts of *Toona sinensis* leaves (TSL-1) post-treatment on NO production and iNOS protein expression in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. TSL-1 (50 μg/mL) was added during or 1 hour, 2 hours, 4 hours, of 6 hours after LPS (1 μg/mL) treatment. (A) The supernatant was collected after 24 hours of LPS treatment for NO analysis. Nitrite levels in cultured media were determined by Griess assay and were reflected to NO levels. Sodium nitrite was used for preparing the standard curve. (B) Total cells lysates were obtained 24 hours after LPS (1 μg/mL) treatment. Equal amounts of cell lysates were analyzed by Western blotting using anti-inducible NO synthase-specific antibody. Densitometric values indicate the relative ratio of inducible NO synthase/β-actin. Data are expressed as a percentage of the LPS alone values. Bars represent means ± standard deviation of six separate experiments. *$p < 0.05$, *** $p < 0.001$ versus the LPS-treated alone group.

correlate the important role of NO in the function of the central nervous system. In the central nervous system, activated microglia are the major cellular source of iNOS. Therefore, the inhibitory effect that TSL-1 exerts on iNOS might be beneficial not only in the protection of neurons but also as therapy through microglia.

The neuroprotective potential of TSL-1 might be due to the biological activities of compounds in the leaf extract. More than eight compounds, including gallic acid, methyl gallate, ethyl gallate, kaempferol, kaempferol-3-O-b-D-glucoside, quercetin, quercitrin, quercetin-3-O-β-D-glucoside, and rutin, have been isolated from TSL-1, as previously described [30,48]. Of these compounds, gallic acid isolated directly from TSL-1 has been demonstrated to possess effective antioxidant activity against various oxidative stress such as leukemia, atherogenesis, prostate cancer, oral carcinoma, and angiogenesis in the liver, kidney, and testis [18,24,29,30,49,50]. Quercetin purified from TSL-1 specifically elevates the activities of antioxidant

enzymes only in the testis [50]. In the central nervous system, gallic acid and quercetin have also been found to reduce neuronal damage by inhibiting microglia-mediated NO release, TNF-α production, and oxidative stress [51–55]. Therefore, it is reasonable to infer the potential therapeutic effect of TSL-1 or its compounds, such as gallic acid or quercetin, on the suppression of inflammatory-related neuronal injury and oxidative stress in neurodegenerative diseases.

The application of traditional Chinese herbs for medicinal use has attracted attention in recent years. Increasing evidence has suggested that Chinese herbs have therapeutic effects on neurodegenerative diseases such as PD and AD through their anti-inflammatory features [56–58]. These traditional Chinese medicines, such as the extracts of *Tripterygium wilfordii* Hook. f. and *Anemarrhena asphodeloides* Bunge, or the more recently studied grape seed extract, have been shown to promote neuronal survival and neurite growth, to facilitate functional recovery after brain injury, and to act as inhibitors of neuroinflammatory toxicity of activated-microglia. However, it has been proposed that an exacerbated inflammatory response was responsible for causing the impairment in the phagocytosis of amyloid protein deposits by microglia in the AD brain. In this connection, the discovery of agents that are capable of increasing amyloid protein uptake by phagocytic cells is of potential therapeutic interest for AD. In fact, with amyloid β treatment, the blockade of chloride intracellular ion channel 1 will stimulate amyloid β phagocytosis in microglia while inhibiting *i*NOS induction and TNF-α production [59,60]. In the present study, pretreatment with TSL-1 has proved protective against LPS-induced microglial activation. Treatment with TSL-1 at 0 hours and 1 hour post-LPS treatment also exhibited similar degrees of antineuroinflammatory effect comparable to that observed with pretreatment. Therefore, it is likely that the attenuation of LPS-stimulated NO release and NO production are at least partially responsible for neuroinflammation of TSL-1. However, the potential effect of TSL-1 in modifying microglial activation with enhancing amyloid β clearance needs to be addressed.

In conclusion, our results indicate that TSL-1 possesses effective anti-inflammatory features, including the suppression of LPS-induced NO production, as well as the synthesis of TNF-α and *i*NOS protein in BV-2 microglial cells. Because therapeutic agents from herbal sources are usually perceived as being natural and devoid of side effects. It is reasonable to consider TSL-1 as another potential therapeutic agent that works by inhibiting the inflammatory response of microglia in neurodegenerative diseases. To confirm its effect, further studies in *in vivo* animal models are necessary.

Acknowledgments

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