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Celastrol inhibits production of nitric oxide and proinflammatory cytokines through MAPK signal transduction and NF-κB in LPS-stimulated BV-2 microglial cells

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Abbreviations: AD, Alzheimer's disease; PD, Parkinson's disease

Abstract

Excessive production of nitric oxide (NO) and proinflammatory cytokines from activated microglia play an important role in human neurodegenerative disorders. Here, we investigated whether celastrol, which has been used as a potent anti-inflammatory and anti-oxidative agent in Chinese medicine, attenuates excessive production of NO and proinflammatory cytokines such as TNF-α and IL-1β in LPS-stimulated BV-2 cells, a mouse microglial cell line. We report here that the LPS-elicited excessive production of NO, TNF-α, and IL-1β in BV-2 cells was largely inhibited in the presence of celastrol, and the attenuation of inducible iNOS and these cytokines resulted from the reduced expression of mRNAs of iNOS and these cytokines, respectively. The molecular mechanisms that underlie celastrol-mediated attenuation were the inhibition of LPS-induced phosphorylation of MAPK/ERK1/2 and the DNA binding activity of NF-κB in BV-2 cells. The results indicate that celastrol effectively attenuated NO and proinflammatory cytokine production via the inhibition of ERK1/2 phosphorylation and NF-κB activation in LPS-activated microglia. Thus, celastrol may be an effective therapeutic candidate for use in the treatment of neurodegenerative human brain disorders.

Keywords: anti-inflammatory agents; cytokines; extracellular signal-regulated MAP kinases; medicine, Chinese traditional; microglia; nitric oxide synthase type II; triterpene

Introduction

Microglia, the resident macrophages of the CNS, serve a role in immune surveillance and host defense (Baroon, 1995). When neuronal cells die, microglia are rapidly activated and produce reactive oxygen species (ROS), NO, and a variety of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Boje and Arora, 1992; Chao *et al.,* 1992; Liu and Hong, 2003; Kim and Joh, 2006). Studies using cell cultures and animal models such as Parkinson's disease (PD) and Alzheimer's disease (AD) have demonstrated that excessive quantities of individual toxic inflammatory mediators produced by activated microglia can contribute to the degeneration of neurons (Chao *et al.,* 1992; McGuire *et al.,* 2001). Thus, controlling microglial activation may have a therapeutic benefit in the treatment of many neurodegenerative diseases.

Celastrol is a pentacyclic-triterpene extracted from *Tripterygium wilfordii Hook*, a perennial creeping plant that is indigenous to a large area in southern China, is known as the "Thunder of God Vine", and belongs to family Celastraceae (Allison *et al.,* 2001). Extracts containing celastrol have been given to Chinese patients for many years without any published reports of carcinogenicity or other limiting side effects (Li *et al.,* 1997; Liu *et al.,* 2003). Celastrol is reported to have anti-inflammatory properties such as inhibitory activity on the release of NO, TNF-α, and IL-1β in LPS-stimulated human monocytes (Pinna *et al.,* 2004) and mouse peritoneal macrophages (Xu *et al.,* 1991; Suh *et al.,* 1998; Lee *et al.,* 2006). However, little is known of the inhibitory effects of celastrol in microglial activation, and several studies have mainly focused on its therapeutic effects in neurodegenerative diseases, including AD and PD (Allison *et al.,* 2001; Cleren *et al.,* 2005). Thus, celastrol seems to be a useful drug for the treat-

ment of neurodegenerative diseases accompanied by inflammation, and an interesting candidate as a modulator of the inflammatory and immune response (Sethi et al., 2007).

Therefore, in the present study, we investigate the pharmacological effect of celastrol on LPS-induced inflammation in BV-2 microglial cells. We also examined the working mechanism of microglial activation by regulation of mRNA transcription, MAPK phosphorylation, and NF-κB activation.

Materials and Methods

Cell culture

The BV-2 cells, a mouse microglia line, were maintained in DMEM supplemented with 5% heat-inactivated FBS (Hyclone, Logan, UT) and 1% streptomycin/penicillin (Gibco, Grand Island, NY) under a humidified atmosphere of 5% CO_2/ 95% air. Prior to each experiment, cells were plated in 24-well plates at a density of 1×10^5 cells for culture supernatant tests or in 6-well plates at a density of 2×10^5 cells for protein extraction and RNA extraction. The next day, the cells were pre-treated with celastrol (Microsource, Gaylordsville, CT) for 30 min as indicated in the figures, followed by stimulation with LPS (serotype O111: B4, 10 ng/ml; Sigma, St Louis, MO) for the indicated amount of time.

Lactate dehydrogenase (LDH) release assay

Cytotoxicity was determined by measuring the release of LDH. BV-2 cells were treated with different concentrations (1 nM, 100 nM, and 10 μM) of celastrol in the absence or presence of LPS (10 ng/ml) for 3, 6, 12, and 24 h, and the culture medium was used to assay LDH activity. The reaction was initiated by mixing 0.1 ml of cell-free supernatant with potassium phosphate buffer containing NADH and sodium pyruvate in a final volume of 0.2 ml in 96-well plates. The absorbance values were read at 490/630 nm on an automated SpectraMAX 340 microtiter plate reader. Data were expressed as the mean LPS-treated cells (100%) vs. control or celatrol-treated cells.

NO release assay

BV-2 cells were pre-treated with various concentrations of celastrol (1, 10, and 100 nM) for 30 min prior to stimulation with LPS (10 ng/ml) for 24 h. The culture media were removed and assayed for nitrite production, a stable metabolite byproduct of NO generation, using the Greiss reagents (1% sulfanilamide / 0.1% N-(1-naphthyl)-ethylenediamine dihydrocholoride / 2.5% phosphoric acid). In brief, 100 μl medium or sodium nitrite standards were transferred to a 96-well plate, followed by the addition of 100 μl of Greiss reagent. The absorbance values were read at 540 nm on an automated SpectraMAX 340 microtiter plate reader. NO concentration was calculated with reference to the standard curve of sodium nitrite generated by known concentrations.

ELISA

The levels of two cytokines, TNF-α and IL-1β, were assessed by ELISA using monoclonal antibodies and the procedure recommended by the supplier (eBiosciences, San Diego, CA). Cells were pre-treated with various concentrations of celastrol (1, 10 and 100 nM) for 30 min prior to stimulation with LPS (10 ng/ml) for 6 h (for TNF-α) and 24 h (for IL-1β). Supernatants were collected and used for the analysis of TNF-α and IL-1β by ELISA. The concentration of each cytokine was calculated according to the standard curve of the ELISA kits.

RNA extraction and RT-PCR

BV-2 cells were pre-treated with 100 nM of celastrol for 30 min prior to stimulation with LPS (10 ng/ml) for 6 h. Total cellular RNA was isolated from cells in 0.5 ml of Trizol reagent (Invitrogen, Carlsbad, CA). After treatment with DNase I (Promega, Madison, WI) for 30 min at 37°C, 1 μg of total RNA was reverse transcribed for 1 h at 42°C in a reaction mixture containing RNA, 5 U RNase inhibitor (Amersham, Piscataway, NJ), 0.5 mM deoxynucleotide triphosphate (dNTP), 0.5 μM oligo-dT primer, 1× reverse transcriptase buffer, and 5 U of Superscript Reverse Transcriptase (Promega, Madison, WI) in a total volume of 20 μl. PCR was performed using the above prepared cDNA as a template with the following cycle parameters: 94°C, 2 min, 30-35 cycles; 94°C, 30 s; 57-62°C, 30 s; 72°C, 1 min; 92°C, 10 min. PCR reaction products were then run on 1% agarose gels at 100 V and visualized with ethidium bromide (EtBr; 0.5 μg/ml). Verification of specific genes was established by assessing their predicted sizes under UV light. The primer sequences are as follows: sense; 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3', anti-sense; 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' for iNOS; sense; 5'-GGCAGGTCTACTTTGGAGTCATTGC-3', anti-sense; 5'-ACATTCGAGGCTCCAGTGAATTCGG-3' for TNF-α; sense; 5'-ATGGCAACTGTTCCTGAACTCAAC-

T-3', anti-sense; 5'-CAGGACAGGTATAGATTCTTT-CCTTT-3' for IL-1β; and 5'-CTCGTGGAGTCTACT-GGTGT-3', 5'-GTCATCATACTTGGCAGGTT-3' for β-actin as a control for PCR.

Western blot analysis

BV-2 cells were pre-treated with 100 nM of celastrol or 30 μM of ERK inhibitor (PD98059; Calbiochem, San Diego, CA) for 30 min prior to stimulation with LPS (10 ng/ml) for 24 h (for iNOS) and 15 min (for ERK1/2). Whole cell extracts were prepared by lysing cells for 30 min on ice in lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 1% NP-40), followed by centrifugation at 12,000 rpm for 10 min. The protein concentration was determined with Lowry protein assay solution (Bio-Rad, Hercules, CA). Twenty micrograms of protein were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes (Hybond ECL, Amersham, Piscataway, NJ). The membranes were blocked with 5% skim milk (Difco, Becton, Dickinson and Company, Sparks, MD) in 10 mM Tris-HCl containing 150 mM NaCl and 0.5% Tween-20 (TBST), and were then incubated overnight with primary antibodies at 4°C, followed by incubation with HRP-conjugated secondary antibodies (Sigma). The blots were developed using an enhanced chemiluminescence detection kit (Amersham). The antibodies used in this study were anti-iNOS (1:1,000, SantaCruz Biotechnology, Santa Cruz, CA), anti-phospho-ERK1/2 (1:1,000, Cell Signaling, Beverly, MA), and anti-β-actin (1:2,000, Sigma).

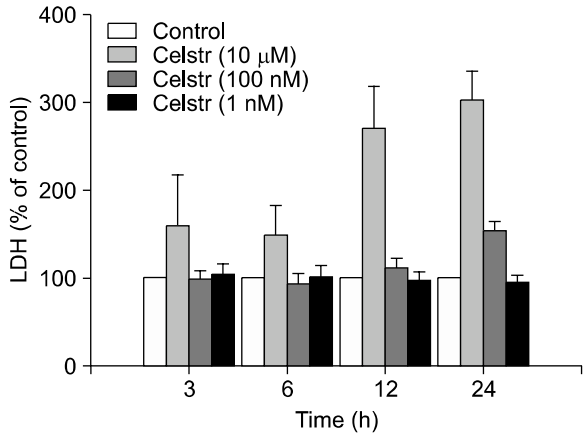

Figure 1. Effect of celastrol on the cytotoxicity in BV-2 cells. Cells were treated with various concentrations of celastrol and incubated for 3, 6, 12, and 24 h. The cytotoxicity of celastrol was measured by LDH release assay. Data represent the mean ± SEM of three independent experiments.

Nuclear extraction and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from BV-2 cells were prepared as follows: cells were treated with 1 ml of lysis buffer on ice for 30 min. After centrifugation at 1,500 × *g* for 10 min, the pellet was resuspended in 50 μl of extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF) and incubated on ice for 30 min. After centrifugation at 1,500 × *g* for 5 min, the supernatant was harvested as the nuclear protein extract and stored at −70°C until use. The protein concentration was determined with Lowry protein assay solution (Bio-Rad, Hercules, CA). Ten micrograms of the nuclear proteins were incubated with ^{32}P-labeled NF-κB probe on ice for 30 min and resolved on 5% acrylamide gel.

Statistical analysis

Experiments were independently performed a minimum of three times, and the data are presented as

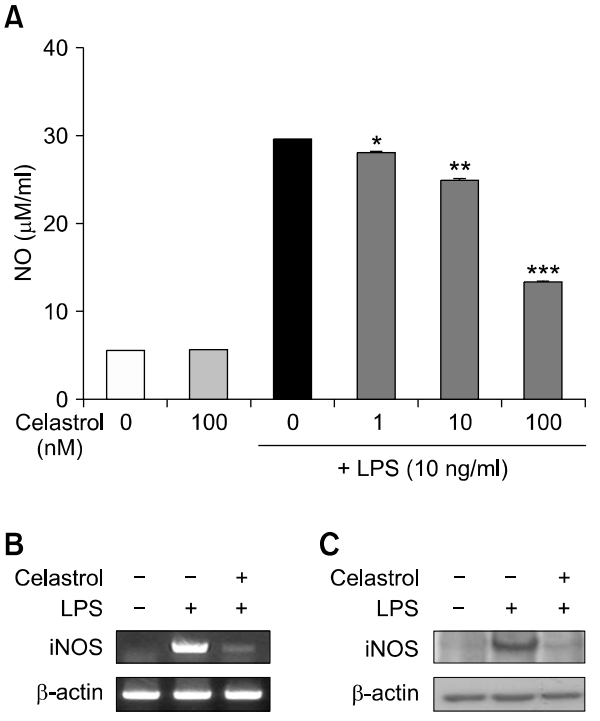

Figure 2. Effect of celastrol on LPS-induced production of NO in BV-2 cells. (A) Cells were treated with celastrol (1, 10, and 100 nM) in the absence or presence of LPS (10 ng/ml) for 24 h. The NO concentration was determined in culture medium using Griess reagent. Data represent the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. LPS alone. (B and C) Cells were treated with celastrol (100 nM) in the absence or presence of LPS for 6 h for RT-PCR and 24 h for Western blot. The mRNA expression and protein levels of iNOS were evaluated by RT-PCR and Western blot.

the mean ± SEM (standard error of the mean). Data were analyzed using Student's *t*-test and one way ANOVA. Statistical significance was established at $P < 0.05$.

Results

Inhibition of NO production by celastrol

To investigate the effects of celastrol on NO production in LPS-stimulated BV-2 cells, cells were treated with LPS alone or with various concentrations of celastrol for 24 h. NO production was determined by measuring the levels of a stable NO metabolite, nitrite, in the culture medium by Griess reaction. At the concentrations used in this study, none of the celastrol treatments caused toxicity to cells, as confirmed by the LDH assay (Figure 1). Celastrol decreased the LPS-induced production of NO in BV-2 cells in a dose-dependent manner (Figure 2A). Next, to elucidate the mechanism responsible for the inhibitory effect of celastrol on NO production, we determined the iNOS mRNA and protein levels by RT-PCR and Western blot analysis. At a concentration of 100 nM, celastrol effectively inhibited iNOS mRNA expression (Figure 2B) and its LPS stimulation-induced protein levels (Figure 2C) in BV-2 cells to a near basal level. These data suggest that celastrol acts principally by regulating the accumulation of NO at the post-transcriptional level, and that it could be a suppressor of microglial activation.

Inhibition of proinflammatory cytokine production by celastrol

To investigate the anti-inflammatory effect of celastrol, we determined the production of proinflammatory cytokines, including TNF-α and IL-1β, by stimulating BV-2 cells with LPS in the presence of celastrol. As shown in Figure 3, the stimulation of microglia by LPS increased the production of TNF-α and IL-1β, and celastrol treatment inhibited their production in BV-2 cells in a dose-dependent manner. To elucidate the mechanism responsible for the inhibitory effect of celastrol on TNF-α and

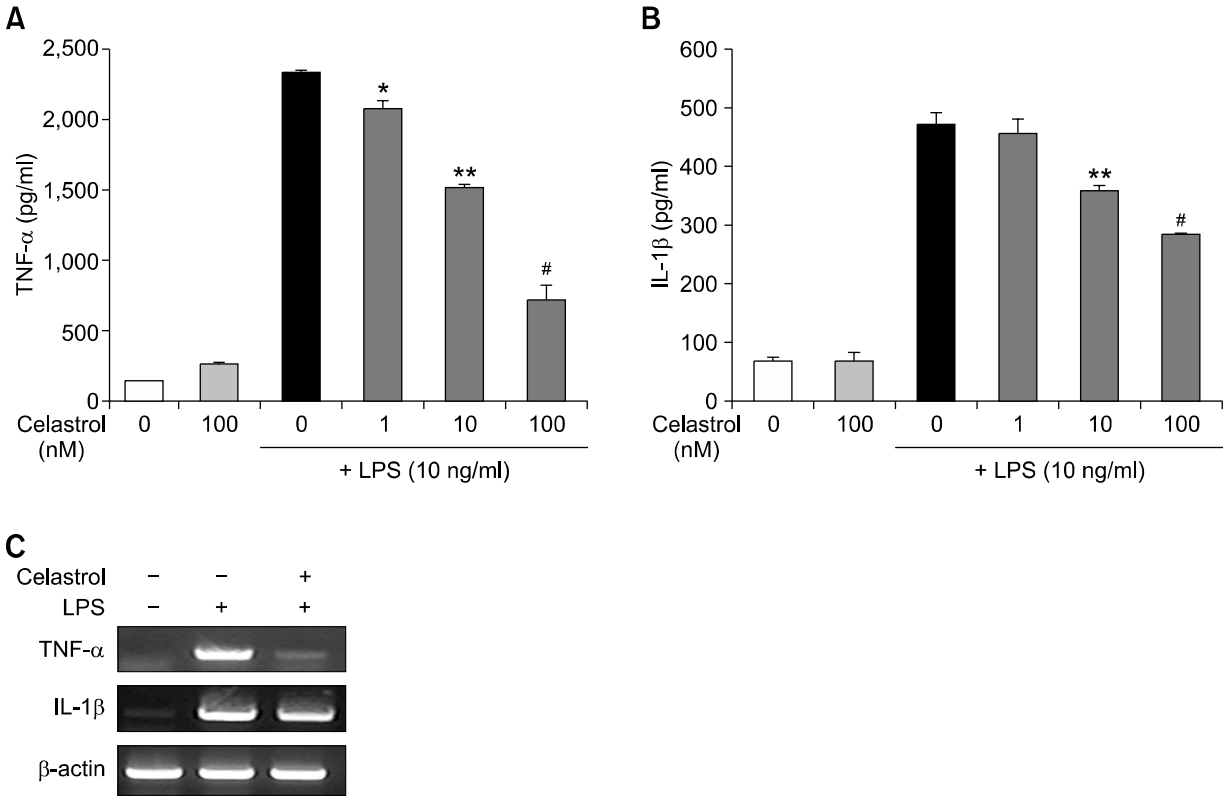

Figure 3. Effect of celastrol on LPS-induced production of proinflammatory cytokine in BV-2 cells. (A) Cells were treated with celastrol (1, 10, and 100 nM) in the absence or presence of LPS (10 ng/ml) for 6 h for RT-PCR and 24 h for ELISA. Each cytokine concentration was measured in culture medium using ELISA. Data represent the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{\#}P < 0.001$ vs. LPS alone. (B) Cells were treated with celastrol (100 nM) in the absence or presence of LPS for 6 h. The mRNA expression of each cytokine was evaluated by RT-PCR.

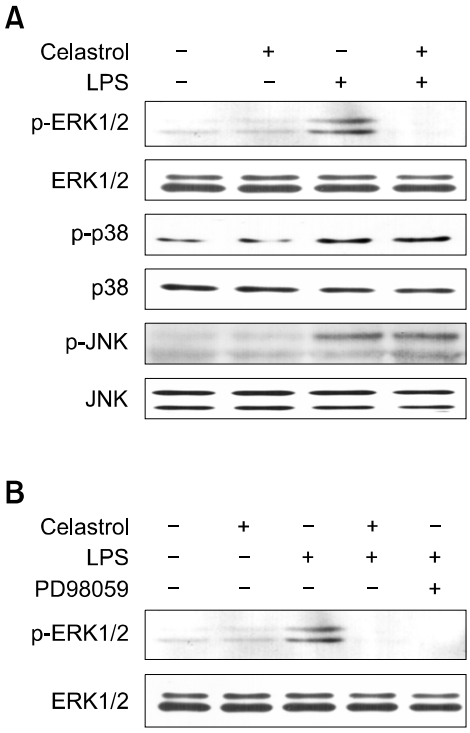

Figure 4. Effect of celastrol on LPS-induced phosphorylation of MAPK molecules in BV-2 cells. (A) Cells were treated with celastrol (100 nM) in the absence or presence of LPS (10 ng/ml) for 15 min. (B) Cells were treated with celastrol (100 nM) or an ERK inhibitor, PD98059 (30 μM), in the absence or presence of LPS for 15 min. Total cell extracts were subjected to immunoblot analysis using antibodies against phosphor- or total forms of three MAPKs.

IL-1β production, we next determined the cytokine mRNA expression levels by RT-PCR analysis. Celastrol markedly decreased the mRNA expression levels of both TNF-α and IL-1β in BV-2 cells (Figure 2A). These data suggest that celastrol might act as a modulator of the accumulation of inflammatory cytokine production at a post-transcriptional level.

Inhibition of ERK1/2 MAPK phosphorylation and NF-κB activation by celastrol

We then assessed whether the repressive effect of celastrol on gene expression occurred via alteration of MAPK activity. As shown in Figure 4, celastrol did not inhibit LPS-induced phosphorylation levels of p38 and pJNK MAPKs in BV-2 cells (Figure 4A). While LPS-induced phosphorylation of ERK1/2 MAPK was greatly inhibited by celastrol and ERK inhibitor (PD98059) in cells, non-phosphorylated ERK remained the same (Figure 4B). These findings indicate that celastrol is effective in the inhibition of ERK phosphorylation in LPS-stimulated microglial cells. We next examined the influence of celastrol on the NF-κB DNA binding activity using EMSA. As shown in Figure 5, celastrol suppressed the LPS-induced DNA binding activity of NF-κB at a concentration of 100 nM in BV-2 cells, which indicated the involvement of the NF-κB pathway.

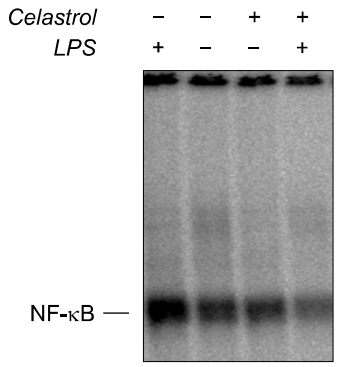

Figure 5. Effects of celastrol on LPS-stimulated NF-κB DNA binding activity in BV-2 cells. Cells were treated with celastrol (100 nM) in the presence or absence of LPS (10 ng/ml) for 1 h. Total nuclear extracts were isolated and used in an electrophoretic mobility shift assay with ^{32}P-labeled NF-κB oligonucleotide as a probe. The arrow indicates the p65 NF-κB binding complex.

Discussion

The various therapies to treat neurodegenerative diseases have specifically included therapeutic modalities aimed to control the pathophysiological mechanisms that lead to the systemic inflammatory response. Microglia are potential sources and targets of relevant neuroprotective factors as well as neurotoxins because their uncontrolled activation may contribute to neurotoxicity in human brain disorders (McGeer *et al.,* 1993; Barron, 1995; Giulian *et al.,* 1996). The activated microglia-derived proinflammatory factors, which include inflammatory cytokines and NMDA receptor antagonists as well as oxygen free radicals and nitric oxide (NO), have long been believed to be involved with neuroinflammation in neurodegenerative diseases, including PD and AD (Boje and Arora, 1992; Chao *et al.,* 1992). Thus, intervention of microglial activation has become a therapeutic target for the treatment of many neurodegenerative conditions (Liu and Hong, 2003; Kim and Joh, 2006). In this study, we studied celastrol, a natural component of *Tripterygium wilfordii Hook*, in LPS-activated BV-2 microglial cells in terms of the production of NO and proinflammatory cytokines, and evaluated the therapeutic potential for treatment of human neurodegenerative disorders.

NO is known to be involved in the processes of CNS inflammation. iNOS is the key enzyme for NO production, and is quantitatively induced in activated glial cells after exposure to stimulators such as LPS and viral infections (McGeer *et al.,* 1993; Chew *et al.,* 2006). Therefore, agents with the ability to inhibit iNOS expression are potentially beneficial in the treatment of conditions associated with an overproduction of NO, including septic shock, inflammation, and neurodegenerative diseases (Hobbs *et al.,* 1999). In the present study, celastrol decreased the NO production induced in LPS-activated BV-2 cells in a dose-dependent manner (Figure 2A), and largely reduced the mRNA expression and protein levels of iNOS in BV-2 cells (Figure 2B and C), which indicated that the inhibition of NO production by celastrol is a result of the inhibition of iNOS gene expression. Indeed, the change of redox status by many antioxidant chemicals in macrophage cells has been reported to directly or indirectly regulate some initiation factors and RNA binding proteins that regulate the translation or stability of iNOS mRNA (Fukuda *et al.,* 1999; Chen *et al.,* 2004).

TNF-α and IL-1 are the two main proinflammatory cytokines produced by activated microglia during inflammation caused by the disruption of the brain-blood barrier (BBB) (Chap *et al.,* 1995; Chew *et al.,* 2006). Celastrol has previously been known to inhibit *in vitro* TNF-α and IL-1β secretion in LPS-activated human peripheral blood monocytes and THP-1 cells (Pinna *et al.,* 2004), as well as in mouse peritoneal macrophage cells (Xu *et al.,* 1991; Suh *et al.,* 1998; Lee *et al.,* 2006). We found that celastrol inhibits LPS-induced production of proinflammatory cytokines, TNF-α and IL-1β (Figure 3A), and the expression of their mRNA (Figure 3B) in BV-2 cells. This result indicates that celastrol is able to modulate the activities of TNF-α and IL-1β at the transcriptional or mRNA stabilization levels in activated microglia.

LPS stimulation induces its inflammatory effects through the activation of both MAPKs signaling and the classical NF-κB pathway (Sanghera *et al.,* 1996). Although the exact intracellular signal pathways of microglial activation by LPS have not been clearly defined, LPS has been reported to induce iNOS and TNF expression in microglial cells through signal pathways, including ERK1/2, p38 MAPK, and NF-κB (Bhat *et al.,* 1998; Shen *et al.,* 2005). Thus, inhibition of these signaling pathways may explain the potent activity of celastrol as a suppressor of inflammatory mediators. Recently, various natural compounds, including baicalein, berberine, curcumin, luteolin, and resveratrol, have been reported to inhibit the overproduction of inflammatory mediators such as NO, COX-2, and TNF-α in LPS-stimulated BV-2 cells (Suk *et al.,* 2003; Kang *et al.,* 2004; Kim *et al.,* 2006) and primary microglia (Chen *et al.,* 2004). Interestingly, these compounds have already been shown to be potent inhibitors of the NF-κB pathway. The present study demonstrated that celastrol largely inhibited ERK1/2 MAPK phosphorylation (Figure 4) and NF-κB DNA binding activity (Figure 5) in LPS-induced microglial activation. This finding suggests that the transcriptional downregulation of the inflammatory mediators by celastrol results from the inhibition of the ERK MAPK and NF-κB signal pathways.

The BBB maintains the homeostasis of the brain microenvironment, which is crucial for neuronal activity and function (Cucullo *et al.,* 2004). Thus, whether various components, including celastrol, might pass through the BBB is important in terms of their potential use in the treatment of many brain disorders. The neuroprotective effects of celastrol in conditions accompanied by inflammation, such as AD and PD in rats and mice, have been described previously (Allison *et al.,* 2001; Cleren *et al.,* 2005), while there is no report or evidence on whether it might pass through the BBB and upregulate dopamine synthesis or inhibit the metabolism of either dopamine or MPP^+. In this study, we did not investigate whether celastrol might pass through the BBB or whether it can exist at detectable tissue concentrations in the brain. In further study, we will test the efficacy of a putative neuroprotective drug, celastrol, using an *in vivo* model of PD.

In conclusion, we demonstrated that celastrol exhibits anti-inflammatory activity through inhibition of inflammatory mediators such as NO, TNF-α, and IL-1β resulting from the downregulation of their gene transcription levels in LPS-activated BV-2 cells. The anti-inflammatory properties of celastrol were mediated by the inhibition of ERK MAPK phosphorylation and NF-κB activation in cells. These data suggest that celastrol is a potent suppressor of LPS-induced inflammatory status in activated microglia, and a potential therapeutic agent for use in the treatment of various neurodegenerative diseases.

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