Suppression of inflammatory responses by celastrol, a quinone methide triterpenoid isolated from Celastrus regelii

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ABSTRACT

Background Celastrol, a quinone methide triterpenoid isolated from the Celastraceae family, exhibits various biological properties, including chemopreventive, antioxidant and neuroprotective effects. In this study, we showed that celastrol inhibits inflammatory reactions in macrophages and protects mice from skin inflammation.

Materials and methods Anti-inflammatory effects of celastrol (0–1 μM) were examined in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. To investigate the effects of celastrol (0–50 μg per mice) in vivo, activation of myeloperoxidase (MPO) and histological assessment were examined in the 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced mouse ear oedema model.

Results Our in vitro experiments showed that celastrol suppressed not only LPS-stimulated generation of nitric oxide and prostaglandin E2, but also expression of inducible nitric oxide synthase and cyclooxygenase-2 in RAW264.7 cells. Similarly, celastrol inhibited LPS-induced production of inflammatory cytokines, including tumour necrosis factor-α and interleukin-6. In an animal model, celastrol protected mice from TPA-induced ear oedema, possibly by inhibiting MPO activity and production of inflammatory cytokines.

Conclusions Our data suggest that celastrol inhibits the production of inflammatory mediators and is a potential target for the treatment of various inflammatory diseases.

Keywords 12-O-tetradecanoyl-phorbol-13-acetate-induced ear oedema, celastrol, cytokine, inflammation.

Introduction

Inflammation is a complex process mediated by activation of various immune cells. Macrophages play a central role in mediating many different immunopathological phenomena during inflammation, including the overproduction of pro-inflammatory cytokines and inflammatory mediators, such as interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α, nitric oxide (NO) synthesized by inducible NO synthase (iNOS) and prostaglandin (PG)E2 synthesized by cyclooxygenase (COX)-2 [1–4].

Common downstream signal transduction regulators in macrophages that have been shown to mediate inflammatory responses include proteins in the nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) families. NF-κB is an essential transcription factor that regulates gene expression for various cytokines, chemokines, growth factors and cell-adhesion molecules [5–7]. In unstimulated cells, NF-κB is constitutively localized in the cytosol as a heterodimer by physical association with an inhibitory protein called inhibitor κB (Iκ-B). Various stimuli, such as lipopolysaccharides (LPS), cytokines, activators of protein kinase C, oxidants and viruses, activate several signal transduction pathways that all lead to phosphorylation and degradation of Iκ-B and subsequent activation of NF-κB [8]. Following activation, the NF-κB heterodimer is rapidly translocated to the nucleus, where it activates the transcription of target genes, including genes encoding for pro-inflammatory cytokines, adhesion molecules, chemokines and inducible enzymes such as COX-2 and iNOS [6,7].

A number of in vitro studies have shown that the production of inflammatory mediators is strongly affected by MAPKs such as p38 MAPK, c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). Phosphorylation of MAPKs is known to be a critical component of NO and pro-inflammatory cytokine production in activated macrophages [9–11]. As
NF-κB and MAPK play a pivotal role in the amplifying loop of the inflammatory response, they have logically become targets for new anti-inflammatory treatments.

There is a growing public interest in the use of complementary and alternative medicine for the prevention and treatment of various diseases [12,13]. Folk medicine, an important component of complementary and alternative medicine, may serve as a useful model for scientific inquiry because it has a standardized system of diagnostics and therapies, and is practiced worldwide [14]. Nortriterpene quinone methides, secondary metabolites of the Celastraceae family, have been used as folk medicines in various countries. They are considered as chemotaxonomic indicators because they are found abundantly in the Celastraceae and Hippocrateaceae families [15]. Tripterygium, one species of Celastraceae family, has been extensively used as a traditional medicine in the treatment of tumours and as an insecticide for hundreds of years [16]. Since the 1980s, many triterpenes have been isolated from this species [17,18], which also contains various diterpenes, sesquiterpenes and alkaloids [19,20]. Among these nortriterpene quinone methides, celastrol is reported to inhibit tumour growth in nude mice with prostate cancer [21]. In addition, multiple assays using animal tumour tissue samples have shown that celastrol inhibits proteasomal activity and induces apoptosis, which suggest that celastrol has great potential for cancer prevention and treatment [22].

Previously, Lee et al. isolated three quinone methide triterpenes – celastrol, pristimerine and 22β-hydroxy-tingenone – from stem bark of Tripterygium regelii and showed that they had antibacterial activity [23]. In this study, we showed that celastrol effectively suppresses the inflammatory responses accompanying the direct inhibition of NO, PGE2 and inflammatory cytokines in murine macrophages. These anti-inflammatory effects of celastrol were also demonstrated in a 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced ear oedema model.

Methods

Materials
Celastrol (Fig. 1a) was prepared using a previously reported method [23]. LPS derived from *Escherichia coli* (0111:B4) was obtained from Sigma-Aldrich (St Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), foetal bovine serum (FBS), penicillin and streptomycin were obtained from Hyclon (Logan, UT, USA). The antibodies (Abs) used in this study included: anti-iNOS rabbit polyclonal, anti-COX-2 mAb (BD Biosciences, San Jose, CA, USA), anti-1xβ2-mAb, anti-phospho-JNK (pT183, pY185) rabbit polyclonal, anti-JNK (rabbit polyclonal, anti-phospho-ERK1/2 (pT202, pY204) rabbit polyclonal, anti-ERK1/2 rabbit polyclonal, anti-phospho-p38 pT180, pY182) rabbit polyclonal, anti-p38 rabbit polyclonal (Cell Signaling Technology, Danvers, MA, USA) and anti-β-actin mAb (Sigma) antibodies.

Cell culture and cell viability assay
RAW264.7 murine macrophages were obtained from the Korean Cell Bank (Seoul, Korea) and cultured in DMEM containing 10% FBS, 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin at 37 °C in 5% CO₂. The effect of celastrol on cell viability was tested using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Cells were plated at a density of 2 × 10⁴ cells in a 96-well flat-bottom plate, and celastrol was added to each well at the indicated concentrations. After 24 h of incubation, cell viability was measured according to the manufacturer’s instructions.

Measurement of nitrite, PGE₂ and cytokines
RAW264.7 cells were plated at a density of 5 × 10⁵ cells in a 24-well plate with 500 µL of culture medium per well, and incubated for 12 h. They were then treated with various doses of celastrol in 1 µg mL⁻¹ of LPS and incubated for another 18 h. The amount of nitrite production was measured using the Griess reagent system (Promega). The amount of PGE₂ production was determined using an enzyme-linked immunosorbent assay.
(ELISA) kit (R & D, Minneapolis, MN, USA), according to the manufacturer’s instructions. The amounts of IL-6 and TNF-α in the cell-free culture supernatants and ear homogenates were measured using an ELISA kit (eBioscience, San Diego, CA, USA).

Western blot analysis
Whole cell extracts (30 µg protein/lane) were separated on 10% sodium dodecylsulphate (SDS)-polyacrylamide gels. The separated proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were then incubated with the indicated antibodies, and specific bands were visualized using an ECL kit (Amersham Biosciences, Boston, MA, USA).

Multiplex analysis of transcription factors
RAW264.7 cells were treated with LPS in the presence of celastrol (0, 0.1, 0.25, 0.5 and 1 µM) for 4 h, and nuclear extracts were prepared. The levels of four transcription factors – NF-κB, activator protein (AP)-1, CCAAT-enhancer box-binding protein (C/EBP) and cyclic AMP-responsive element-binding protein (CREB) – were analysed using the multiplex Procarta Transcription Factor Assay Kit (Panomics, Fremont, CA, USA) [24]. Briefly, nuclear extracts were normalized for protein content and incubated with a mixture of biotin-labelled, double-stranded oligonucleotide probes to form protein/DNA complexes. Unbound probes were then removed by filtration, followed by elution of the complexes from the filter matrix. Bound probes were denatured and hybridized with transcription factor-specific antisense oligonucleotide-conjugated microbeads. Probe-bound microbeads were detected with streptavidin-conjugated R-phycoerythrin and analysed using the Bio-Plex Luminex 100 system (Bio-Rad, Hercules, CA, USA).

AP-1 and NF-κB activity
Nuclear extracts obtained for the multiplex analysis of transcription factors were incubated in microwells coated.

**Figure 2** Effect of celastrol on LPS-induced NO and PGE2 production, as well as on iNOS and COX-2 expression. RAW264.7 cells were treated with 0–1 µM of celastrol in the presence of LPS or vehicle alone for 18 h, and NO (a) and PGE2 (b) production were determined. The results are reported as mean values ± SEM of four independent experiments in triplicate. (c) RAW264.7 cells were treated similarly as in Fig. 2a. Total cell lysates (30 µg of proteins) were resolved on SDS–polyacrylamide gel electrophoresis for iNOS and COX-2 determination. β-actin expression is shown as a loading control. Statistical significance is based on the difference when compared with LPS-stimulated cells (**P < 0.01, ***P < 0.001). ###Significant difference between vehicle and LPS treatment alone, P < 0.001.
with probes containing the NF-κB and AP-1 consensus binding sequence. Transcriptional activities were measured using TransAM (Active Motif, Carlsbad, CA, USA), according to the manufacturer’s instructions.

TPA-induced mouse ear oedema model

Protocols for animal experiments were approved by the Ethics Committee of the Animal Center at Hallym University (Chuncheon, Korea). Six-week-old male ICR mice (Orient Bio Inc, Seongnam, Korea) were randomly assigned to one of five groups (seven mice in each group) and maintained under specific pathogen-free conditions at the animal facility of Hallym University.

To induce inflammation, TPA dissolved in acetone (2.5 μg per ear) was applied topically to the inner and outer sides of the ear of each mouse every 24 h for 3 days. Celastrol (0, 5, 10 and 50 μg per ear) or dexamethasone (50 μg per ear) was administered topically for three consecutive days to the ears of mice 1 h after TPA painting. Ear thickness was measured using a digital thickness gauge (Mitutoyo Corporation, Kawasaki-Shi, Japan). Six hours after the last TPA stimulus, a 5-mm ear pad biopsy was collected from the ear of each mouse with a coring tool (Natsume Seisakusho, Tokyo, Japan) and weighed to determine the severity of oedema. After weighing, biopsies were fixed in 10% neutral buffered formalin, stained with haematoxylin and eosin, and examined histologically.

Part of the ear was homogenized in 50 mM Tris–HCl buffer (pH 7.5) with 1 mM EDTA, and the homogenate was incubated on ice for 20 min in the presence of 0.1% Triton X-100. The homogenate was centrifuged at 10 000 × g for 15 min, and the supernatant was collected for cytokine measurement.

To measure myeloperoxidase (MPO) activity, we homogenized each ear sample in 80 mM sodium phosphate buffer (pH 5.4) containing 0.5% hexadecyl trimethylammonium bromide for 45 s at 4 °C, then centrifuged the sample at 11 200 × g for 20 min at 4 °C. In the measurement of MPO activity, a reaction solution (200 μL) containing 100 μL of 80 mM sodium phosphate buffer, 85 μL of 0.22 M sodium phosphate buffer and 15 μL of 0.017% H2O2 was added to the supernatant (30 μL). The reaction was started by the addition of 20 μL of 18.4 mM tetramethylbenzidine in 8% dimethylformamide. The reaction mixture was incubated for 3 min at 37 °C and then placed on ice. The reaction was stopped through the addition of 30 μL of 1.46 M sodium acetate buffer (pH 3.0). The enzyme activity of MPO was determined colorimetrically based on absorbance at 620 nm using a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Results are expressed as mean values ± SEM. Figures were evaluated using one-way ANOVA, followed by Duncan’s multiple range tests. GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for all calculations. P-values < 0.05 were considered statistically significant.

Results

Celastrol inhibits the production of inflammatory mediators

To evaluate the anti-inflammatory effects of celastrol, we used an in vitro model with murine RAW264.7 macrophages. As celastrol showed no cytotoxicity at concentrations up to 1 μM in RAW264.7 macrophages (Fig. 1b), we used celastrol at a concentration of 0–1 μM for the rest of the experiments. Initially, we determined the suppressive effect of celastrol

![Figure 3](https://www.ejci-online.com)

**Figure 3** Effect of celastrol on LPS-induced inflammatory cytokine production in murine macrophages. RAW264.7 cells were treated with 0–1 μM of celastrol in the presence of 1 μg mL⁻¹ LPS or with vehicle alone for 24 h. The cell culture media were then collected, and the amounts of TNF-α (a) and IL-6 (b) released within them were measured as described in the Methods section. The results are reported as mean values ± SEM of three independent experiments in triplicate. Statistical significance is based on the difference when compared with LPS-stimulated cells (**P < 0.001), ###Significant difference between vehicle and LPS treatment alone, P < 0.001.
on the LPS-stimulated release of inflammatory mediators, NO and PGE\(_2\) in RAW264.7 cells. The nitrite concentrations in LPS-stimulated RAW264.7 cells and in cells exposed to 0.05 μM celastrol were 27.7 ± 3.9 and 3.2 ± 2.9 μM respectively (Fig. 2a). The inhibitory effects of celastrol on PGE\(_2\) production in LPS-exposed cells were similar to their effects on NO production (Fig. 2b). Consistent with the findings related to NO and PGE\(_2\) production, Western blot analysis showed that LPS-induced iNOS and COX-2 induction in RAW264.7 cells was also reduced by celastrol treatment in a dose-dependent manner (Fig. 2c). This indicates that the celastrol-induced reduction in iNOS and COX-2 expression was responsible for the inhibition of NO and PGE\(_2\) production.

We next sought to determine if celastrol reduced the release of pro-inflammatory cytokines. The release of TNF-α and IL-6 was dramatically suppressed by celastrol co-treatment of LPS-exposed RAW264.7 cells (Fig. 3). These observations indicate that celastrol has evocative anti-inflammatory effects on murine macrophages.

**Reduced release of inflammatory mediator by celastrol is regulated by NF-κB and MAPK**

We further investigated the mechanism by which celastrol inhibits the production of inflammatory mediators such as NO and PGE\(_2\), as well as pro-inflammatory cytokines. As NF-κB and MAPK are major molecules involved in the release of proteins that mediate the inflammatory response [5–11], we evaluated the effect of celastrol on NF-κB and MAPK activation to determine if it is mediated by Iκ-Bα degradation and if it is mediated by MAPK phosphorylation respectively. We found that LPS-induced Iκ-Bα degradation and phosphorylation of ERK and JNK were inhibited after 30 min of exposure to celastrol in a dose-dependent manner, without a change in total protein expression (Fig. 4). However, phosphorylation of p38 was not affected by celastrol treatment (Fig. 4).

**Profiling celastrol-suppressed transcription factors in LPS-stimulated RAW264.7 cells**

Extracellular stimuli related to the inflammatory response are believed to trigger signalling pathways that ultimately lead to the activation of various transcription factors, such as NF-κB, AP-1, C/EBP, and CREB [25–27]. Indeed, the iNOS and COX-2 genes have binding sites for C/EBP, CREB, and NF-κB, which are known to be involved in the LPS/cytokine-mediated induction of transcription [25,26]. To determine the transcription factors affected by celastrol in LPS-stimulated RAW264.7 cells, we performed multiplex analysis of transcription factors. The LPS-induced increase in the activity of NF-κB and AP-1 appeared more dramatic than that of C/EBP and CREB in RAW264.7 cells (Fig. 5a). Celastrol (up to 0.5 μM) treatment significantly reduced the activation of NF-κB and AP-1, but not of C/EBP or CREB.

To confirm the effect of celastrol on the nuclear translocation and DNA binding of NF-κB and AP-1, we performed a transcription factor ELISA assay. We found that LPS-induced DNA binding of NF-κB and AP-1 was significantly inhibited in

**Figure 4** Effect of celastrol on LPS-induced Iκ-Bα degradation and MAPK phosphorylation in murine macrophages. RAW264.7 cells were plated in 100-mm dishes. After 12 h of seeding, cells were treated with different doses of celastrol for 1 h, followed by stimulation with 1 μg mL\(^{-1}\) of LPS for 30 min. Whole cell extracts were prepared for Western blotting with the indicated antibodies. β-actin was used as a control. Statistical significance is based on the difference when compared with LPS-stimulated cells (*P < 0.05, ***P < 0.001). ###Significant difference between vehicle and LPS treatment alone, P < 0.001.
LPS-stimulated RAW264.7 cells exposed to celastrol, in a dose-dependent manner (Fig. 5b). These observations indicate that celastrol exerts anti-inflammatory effects via inhibition of NF-κB and AP-1 activity.

**Effect of celastrol on TPA-induced ear oedema**

Evaluation of the in vivo anti-inflammatory activity of celastrol was performed in a TPA-induced mouse ear oedema model.

Ear oedema was measured in the ears prior to and every 24 h following topical application of TPA. As shown in Fig. 6a,b, exposure to TPA resulted in marked increases in skin thickness and weight. Topical application of the vehicle (acetone) did not significantly alter the skin thickness or weight. However, celastrol and dexamethasone (an anti-inflammatory drug) significantly inhibited TPA-induced increases in ear thickness and weight, indicating the therapeutic effect.
The inflamed auricles of the mice were examined histologically after haematoxylin and eosin staining (Fig. 6c). The epidermises of the TPA-treated, vehicle-administered mice showed hyperplasia. The dermis showed significant oedema and infiltration of mononuclear cells. Celastrol markedly suppressed TPA-induced epidermal hyperplasia to a degree similar to that seen in dexamethasone-treated mice. Finally, this topical anti-inflammatory activity was also confirmed by measuring MPO activity, a biochemical marker of neutrophil infiltration in ear biopsies [28]. The TPA-induced elevation in MPO activity was also significantly attenuated by treatment with celastrol (Fig. 7a). In addition to MPO activity, treatment with TPA plus celastrol or dexamethasone reduced IL-6 levels significantly (Fig. 7b).

Discussion

The search for natural products with anti-inflammatory activity has increased enormously in recent years. Celastrol, a quinone methide triterpenoid, has been identified as having a broad spectrum of biological activities [19–23], but few reports have examined the potential anti-inflammatory actions of celastrol and the mechanisms involved. In this study, we examined the effects of natural celastrol on selected macrophage functions in an attempt to define its potential as an anti-inflammatory agent. We noted that low concentrations (0.05–1 µg per ear) of celastrol inhibited the production of NO and PGE2 in LPS-stimulated macrophages (Fig. 2). These inhibitory effects were accompanied by concentration-dependent decreases in the expression of iNOS and COX-2 protein, indicating that the reduced release of NO and PGE2 can be attributed to the inhibition of iNOS and COX-2 expression. Supporting these data, celastrol efficiently inhibited cytokine release (TNF-α and IL-6) by LPS-stimulated macrophages (Fig. 3) and reduced inflammation and neutrophil infiltration in an in vivo mouse model of ear oedema (Figs 6 and 7).

As NF-κB, AP-1, C/EBP and CREB are important transcription factors that regulate a number of genes during inflammation [6,7,11,27,29,30], we tried to identify specific transcription factors affected by celastrol in LPS-stimulated murine macrophages. This study showed that celastrol negatively influences the activity of NF-κB and AP-1, but not that of C/EBP or CREB.
Three families of MAPKs – ERK, JNK and p38 MAPK – play a critical role in the regulation of cellular responses to cytokines and stressors. In addition, MAPKs are also important signalling molecules that regulate gene transcription and mRNA expression of pro-inflammatory cytokines/mediators in LPS-treated RAW 264.7 cells. Serum and growth factors induce AP-1 by activating ERK. The induction of AP-1 by pro-inflammatory cytokines is principally mediated by JNK and p38 MAPK. Stimulation of macrophages by LPS activates all three MAPK pathways, and the activation of these signalling pathways in turn activates a variety of transcription factors, such as AP-1 and NF-κB. In addition, Kang et al. used specific inhibitors of MAPK to show that all MAPKs play an important role in the regulation of AP-1 activity in RAW 264.7 cells [11]. In this study, celastrol inhibited the LPS-induced transcriptional activity of AP-1 and phosphorylation of JNK and ERK. Considering the importance of AP-1 and MAPK in the regulation of the inflammatory response, these results suggest that the suppression of inflammatory mediators by celastrol is mediated via the inhibition of AP-1, JNK and ERK activity. To our knowledge, this study is the first to demonstrate the inhibitory effect of celastrol on the activity of AP-1 and MAPK in RAW264.7 cells.

In conclusion, we demonstrated that celastrol strongly inhibits the production of inflammation-associated mediators (NO, PGE2, TNF-α and IL-6) at low concentrations. This mechanism may be mediated via suppression of NF-κB and AP-1 activation. Our study suggests that celastrol is a potential treatment for inflammatory diseases in humans.

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