

Celastrol inhibits IL-1 β -induced inflammation in orbital fibroblasts through the suppression of NF- κ B activity

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Abstract. Graves' disease is an autoimmune disease of the thyroid gland, which is characterized by hyperthyroidism, diffuse goiter and Graves' ophthalmopathy (GO). Although several therapeutic strategies for the treatment of GO have been developed, the effectiveness and the safety profile of these therapies remain to be fully elucidated. Therefore, examination of novel GO therapies remains an urgent requirement. Celastrol, a triterpenoid isolated from traditional Chinese medicine, is a promising drug for the treatment of various inflammatory and autoimmune diseases. CCK-8 and apoptosis assays were performed to investigate cytotoxicity of celastrol and effect on apoptosis on orbital fibroblasts. Reverse transcription-polymerase chain reaction, western blotting and ELISAs were performed to examine the effect of celastrol on interleukin (IL)-1 β -induced inflammation in orbital fibroblasts from patients with GO. The results demonstrated that celastrol significantly attenuated the expression of IL-6, IL-8, cyclooxygenase (COX)-2 and intercellular adhesion molecule-1 (ICAM-1), and inhibited IL-1 β -induced increases in the expression of IL-6, IL-8, ICAM-1 and COX-2. The levels of prostaglandin E2 in orbital fibroblasts induced by IL-1 β were also suppressed by celastrol. Further investigation revealed that celastrol suppressed the IL-1 β -induced inflammatory responses in orbital fibroblasts through inhibiting the activation of nuclear factor (NF)- κ B. Taken together, these results suggested that celastrol attenuated the IL-1 β -induced pro-inflammatory pathway in orbital fibroblasts from patients with GO, which was associated with the suppression of NF- κ B activation.

Introduction

Graves' disease (GD) is an autoimmune disease of the thyroid gland, which is characterized by hyperthyroidism, diffuse goiter and Graves' ophthalmopathy (GO) (1). It has been reported that up to 50% of patients with GD develop the ocular complication, GO (2,3). The principle characteristics of GO include upper eyelid retraction, soft tissue swelling, proptosis, strabismus, erythema of periorbital tissues, and compressive optic neuropathy, and certain patients with GO suffer from inflammation, diplopia, intense pain, and compressive optic neuropathy or corneal ulceration, which threaten vision (1,4).

Although the pathogenesis of GO remains to be fully elucidated, it is widely accepted that the occurrence of this disease is associated with the abnormal secretion of inflammatory cytokines (1,5,6). These overexpressed inflammatory cytokines promote the infiltration of thyroid lymphocytic and the activation of B cells, which result in the production of autoimmune antibodies against thyroid antigens and contribute to the pathogenesis of GO. It has been demonstrated that, when stimulated by proinflammatory cytokines, orbital fibroblasts from patients with GO can produce excess glycosaminoglycans and inflammatory cytokines, including interleukin (IL)-6, and IL-8 (7-9). The expression levels of intercellular adhesion molecule-1 (ICAM-1) (10,11) and cyclooxygenase (COX)-2 (12,13) have also been found to upregulated in the orbital connective tissues of patients with GO. Currently, glucocorticoids are used as the first-line treatment for GO due to their marked anti-inflammatory and immunosuppressive effects. However, although glucocorticoids are effective in a substantial number of patients with GO, they have several long-term side effects, including hypertension, diabetes and osteoporosis (14). Therefore, it is essential to investigate novel therapies for the management of GO.

Traditional medicines offer an abundance of plant-derived remedies to identify novel lead molecules for the development of novel drugs. Celastrol is a pentacyclic triterpenoid, which was originally isolated from Thunder God Vine root. Celastrol has been demonstrated to exert potent inhibitory action on tumorigenesis. Several studies have reported that celastrol inhibits the proliferation of a variety of tumor cells and suppresses tumor initiation, promotion and metastasis

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in various cancer models *in vivo* (15,16). In addition, celastrol has potent anti-inflammatory effects, and the efficacy of celastrol as an anti-inflammatory drug has been examined in several diseases, including rheumatoid arthritis (17,18), allergic asthma (19), systemic lupus erythematosus (20) and skin inflammation (21). The nuclear factor (NF)- κ B signaling pathway is well integrated with other signaling pathways, and is important in a number of diseases, including cancer and inflammatory diseases (22,23). Several studies have revealed that celastrol is an inhibitor of the NF- κ B signaling pathway, and that the mutation of cysteine 179 in the activation loop of inhibitor of κ B (I κ B) kinase β (IKK β) eliminates sensitivity towards to celastrol, suggesting that celastrol suppresses NF- κ B activation by targeting cysteine 179 in the IKK (24,25).

In the present study, the effect of celastrol on IL-1 β -induced inflammation was examined in orbital fibroblasts from patients with GO. It was found that celastrol significantly attenuated the expression levels of IL-6, IL-8, COX-2 and ICAM-1, and inhibited the IL-1 β -induced increases in the expression levels of IL-6, IL-8, ICAM-1 and COX-2. It was also demonstrated that the level of prostaglandin E (PGE)₂ in the orbital fibroblasts induced by IL-1 β was suppressed by celastrol. Further investigation revealed that celastrol suppressed IL-1 β -induced inflammatory responses in the orbital fibroblasts through inhibiting the activation of NF- κ B activation. Taken together, the results of the present study suggested that celastrol attenuated the IL-1 β -induced pro-inflammatory pathway in orbital fibroblasts from patients with GO, which was associated with the suppression of NF- κ B activation.

Materials and methods

Reagents. Celastrol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY, USA). Penicillin, and gentamycin were purchased from Amresco, Inc. (Framingham, MA, USA). The Cell Counting Kit-8 (CCK-8) assay kit was obtained from Dojindo Laboratories (Kumamoto, Japan). The BAY-11-7082 and Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). IL-6, IL-8, IL-10 and PGE₂ ELISA Duoset kits, and recombinant human IL-1 β were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

Cell culture. Orbital fibroblasts were cultured from adipose connective tissues, which were obtained from four patients with GO (two male, two female) and severe proptosis associated with increased orbital fat volume during a process of surgical decompression. The control tissues were obtained from two patients with no history of GO or autoimmune thyroid disease, and were collected during the course of upper lid blepharoplasties from 2 individuals (one male, one female). The mean age of all subjects was 58 years. The protocol for obtaining orbital adipose connective tissue was approved by the Institutional Review Board of Longhua Hospital (Shanghai, China), and written informed consent was obtained from all patients.

GO orbital tissues samples were minced and plated directly into culture dishes. The cells were maintained in DMEM containing 10% FBS, penicillin (100 U/ml) and gentamycin

(20 mg/ml), in a humidified 5% CO₂ incubator at 37°C. When the fibroblasts had grown to 80% confluence, the cell culture medium was removed and the cells were washed with phosphate-buffered saline (PBS). The fibroblasts were then passaged serially by treatment with trypsin (Sigma-Aldrich). The cell culture medium was replaced every 2 days, and cells between the third and seventh passage were used for the subsequent examinations.

Cell viability assays. Cell viability was assayed using the CCK-8 according to the manufacturer's protocol. Briefly, 100 μ l cells were seeded onto 96-well plates (1 \times 10⁴ cells/ml) for 24 h, following which the cells were treated with, or without, 1 μ M celastrol for 24 h at 37°C. Subsequently, 10 μ l of the CCK-8 solution was added to each well of the plate, followed by 1 h incubation at 37°C. The optical density (OD) was measured at 450 nm using a microplate reader (Multiskan MK3; Thermo Fisher Scientific GmbH., Darmstadt, Germany). The cell inhibitory rate was calculated according to the following equation: Cell inhibitory rate = [1 - (OD experiment - OD blank) / (OD control - OD blank)] \times 100%. All experiments were performed in triplicate and repeated three times independently.

Apoptosis assays. Apoptosis assays were performed according to the manufacturer's protocols. Briefly, the cells in the logarithmic growth phase were collected and washed with isotonic PBS, following which 1 \times 10⁶ cells were seeded into 6-well cell culture plates. After 24 h, the cell cultures were removed, and the cells were incubated with serum-free DMEM, with or without 1 μ M celastrol, for another 24 h at 37°C. The cells were then digested with trypsin and collected by centrifugation at 300 \times g for 10 min at 4°C. The cells were washed with ice-cold PBS, and resuspended gently with 195 μ l annexin V-FITC binding buffer and 5 μ l annexin V-FITC, following which 10 μ l propidium iodide (PI) solutions were added. The mixture was incubated in the dark at room temperature for 15 min. Cytometric analysis was performed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data acquisition and analysis were performed using the WinMDI 2.9 computer program (BD Biosciences).

Western blot analysis. The cells were collected and washed with ice-cold PBS, following which the cells were centrifuged at 300 \times g for 5 min at 4°C, and the supernatant was removed. The cells were lysed with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) at 4°C for 20 min. The lysates were centrifuged for 10 min at 12,000 \times g at 4°C, and the supernatant was collected. The protein concentration was determined using a Bradford assay (BioRad Laboratories, Inc., Hercules, CA, USA). A total of 30-50 μ g proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% (w/v) gels (Beyotime Institute of Biotechnology), and were then electrophoretically transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Following blocking with blocking buffer (Beyotime Institute of Biotechnology) for 1 h at room temperature, the membrane was incubated with the indicated primary antibodies overnight at 4°C. This was followed by incubation in horseradish peroxidase (HRP)-conjugated corresponding secondary antibodies for 1 h at room temperature. Positive signals were visualized using ECL Advanced Solution (Bioworld Technology, Inc., St. Louis Park,

MN, USA). Actin was used as a loading control. The primary antibodies used in the present study were as follows: Rabbit polyclonal ICAM-1 (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA; cat. no. 4915), rabbit monoclonal COX-2 (1:1,000; Cell Signaling Technology, Inc.; cat. no. 12282) and rabbit monoclonal β -actin (1:1,000; Cell Signaling Technology, Inc.; cat. no. 8457).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNAs were isolated from the orbital fibroblasts using TRIzol reagent (Thermo Fisher Scientific, Inc.). The total RNAs were reverse transcribed into cDNA using Reverse Transcriptase M-MLV (Takara Bio, Inc., Otsu, Japan) and were amplified using SYBR Green Master mix (Takara Bio, Inc.). The mRNA expression was analyzed using an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative gene expression levels were obtained following normalization with β -actin. The thermocycling conditions used were as follows: 95°C for 20 sec; 40 cycles of 95°C for 20 sec, 60°C for 30 sec and 72°C for 30 sec. All reactions were run in triplicate. The primer sequences used were as follows: IL-6, forward 5'-ATGAACCTCTCTCCACAAG-3' and reverse 5'-TGTCAATTTCGTTCTGAAGAG-3' (26); IL-8, forward 5'-GTGCAGTTTTGCCAAGGAGT-3' and reverse 5'-TAATTTCTGTGTTGGCGCAG-3' (26); IL-10, forward 5'-CTTCGAGATCTCCGAGATGCCTTC-3' reverse 5'-ATTCTTCACCTGCTCACGGCCTT-3' (27); ICAM-1, forward 5'-CTCAGTCAGTGTGACCGCAGA-3' and reverse 5'-CCCTTCTGAGACCTCTGGCTTC-3' (28); COX-2, forward 5'-GCTCAAACATGATGTTTCATTG-3' and reverse 5'-GCTGGCCCTCGCTTATGA-3' (29); and β -actin, forward-TCACCCACACTGTGCCCAT-3' and reverse 5'-TCCTTAATGTACGCACGATTT-3' (29). The $2^{-\Delta\Delta C_q}$ method was used to quantify the results (30).

ELISA. The orbital fibroblasts (1×10^6) were seeded into 6-well cell culture plates and, after 24 h, the cell culture medium was replaced with DMEM containing 1% FBS, and 10 ng/ml IL-1 β was added, with or without 1 μ M celastrol. Following 24 h of incubation, the supernatants from the cell cultures were collected, and the concentrations of IL-6, IL-8, IL-10 and PEG-2 were determined using an ELISA kit, according to the manufacturer's protocol. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA).

Luciferase assays. For the luciferase assays, HEK 293T cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were seeded into a 24-well plate at a density of 3×10^4 cells/well. After 24 h at 37°C, HEK 293T cells were transfected with 200 ng firefly luciferase reporter gene construct (per well) and 1 ng pRL-SV40 *Renilla* luciferase constructs (per well) for normalization, using cotransfection with 2.4 μ l Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 24 h post-transfection, the cells were stimulated with 100 ng/ml lipopolysaccharide (Sigma-Aldrich), with or without 1 μ M celastrol, 4 h. Cells were subsequently collected and luciferase activity was measured with the Dual-Luciferase[®] Reporter (DLR[™]) assay system (Promega, Madison, WI, USA).

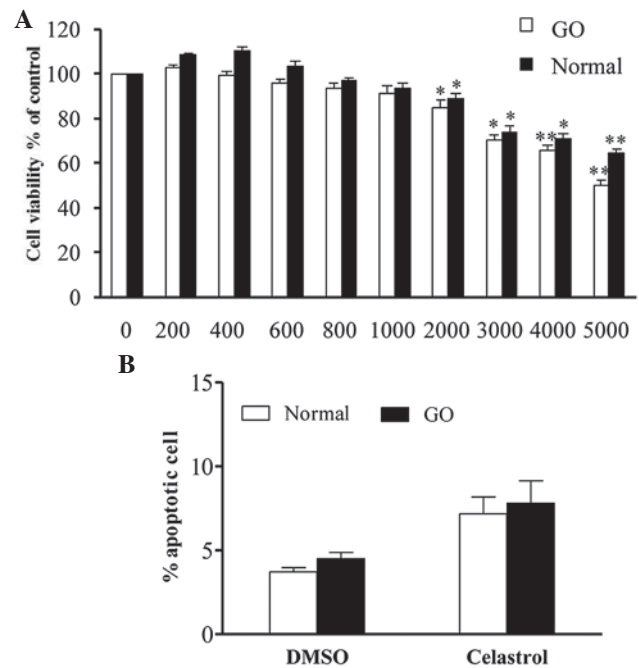


Figure 1. Effect of celastrol on cell viability and apoptosis in orbital fibroblasts. (A) Orbital fibroblasts from normal controls and patients with GO were seeded onto 48-well cell culture plates and treated with different concentrations of celastrol, as indicated, for 24 h. Cell Counting Kit-8 assays were performed to assess cell viability. (B) Effects of celastrol on apoptosis of orbital fibroblasts. The cells were treated with celastrol (1 μ M) or DMSO for 24 h, washed with ice-cold phosphate-buffered saline, resuspended with 195 μ l annexin V-FITC binding buffer and 5 μ l annexin V-FITC, followed by the addition of 10 μ l propidium iodide. The mixture was incubated in the dark at room temperature for 15 min, and cytometric analysis was performed. Assays were performed at least three times. The data are presented as the mean \pm standard error of the mean of three independent experiments. * $P < 0.05$; ** $P < 0.01$ vs. the untreated cells. GO, Graves' ophthalmopathy; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate.

Statistical analysis. All experiments were performed at least three times and the results are presented as the mean \pm standard error of the mean. Student's *t*-test was used to compare two independent groups using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of celastrol on the viability and apoptosis of orbital fibroblasts. In the present study, orbital fibroblasts obtained from normal controls or patients with GO were treated with different concentrations of celastrol (0, 200, 400, 600, 800, 1, 2, 3, 4 and 5 μ M) for 24 h, and cell viability was examined using a CCK-8 assay. As shown in Fig. 1, exposure of the orbital fibroblasts from the GO and normal groups to celastrol at concentrations ≤ 1 μ M for 24 h led to no significant decline in the numbers of living cells, whereas 2 μ M celastrol decreased cell viability in the two groups to 85.02 and 88.94%, respectively (Fig. 1A). The results of the apoptosis assay also showed that exposure of the cells to celastrol at 1 μ M for 24 h did not induce cell apoptosis (Fig. 1B). Therefore, in the subsequent experiments, the cells were treated with 1 μ M celastrol for 24 h to further investigate the role of celastrol in GO.

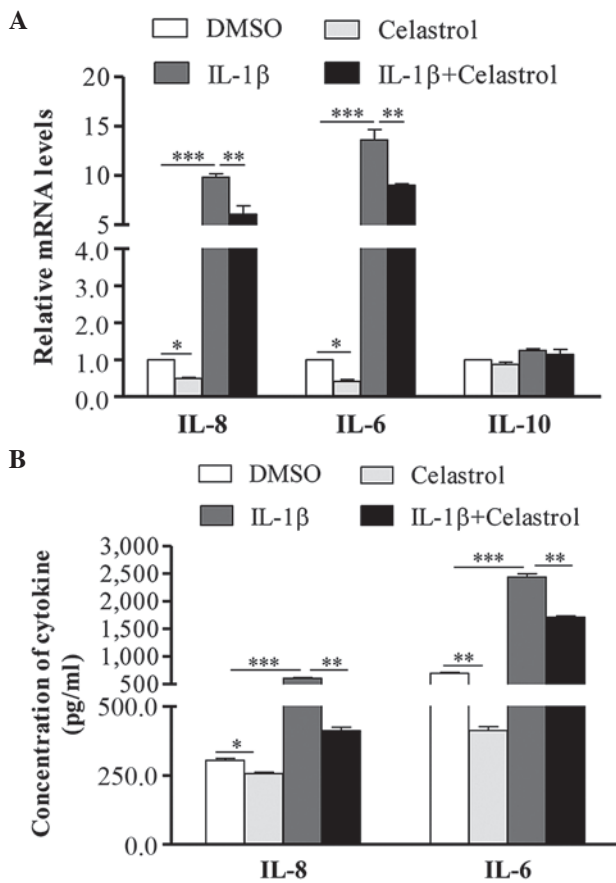


Figure 2. Celastrol suppresses the expression of IL-1 β -induced IL-6 and IL-8 in orbital fibroblasts. Orbital fibroblasts from patients with GO were stimulated with IL-1 β (10 ng/ml), with or without 1 μ M celastrol, for 24 h. (A) Cells were collected and the expression levels of IL-6, IL-8 and IL-10 were determined using reverse-transcription-quantitative polymerase chain reaction analysis. (B) Protein concentrations of IL-6, IL-8 and IL-10 were examined using ELISA. The data are presented as the mean \pm standard error of the mean of three independent experiments. * P <0.05; ** P <0.01; *** P <0.01. DMSO, dimethyl sulfoxide; IL, interleukin.

Effect of celastrol on the expression levels of IL-1 β -induced IL-6 and IL-8. As is already known, inflammation is critical in the pathogenesis of GO, therefore, the present study examined the expression levels of IL-6, IL-8 and IL-10 in IL-1 β -induced GO cells and normal cells, which were treated with or without celastrol. As shown in Fig. 2A, following treatment with IL-1 β , the mRNA expression levels of IL-6 and IL-8 were significantly increased in the GO cells, whereas no change was observed in the expression of IL-10. Celastrol was found to decrease the mRNA expression levels of IL-6 and IL-8 in the IL-1 β -induced orbital fibroblasts. The results of the ELISA also showed that, following stimulation with IL-1 β , the levels of IL-6 and IL-8 in the orbital fibroblast supernatant were significantly upregulated, and co-treatment of celastrol significantly attenuated the IL-1 β -induced expression of IL-6 and IL-8 (Fig. 2B).

Effect of celastrol on the expression levels of IL-1 β -induced ICAM-1 and COX-2. To investigate the effect of celastrol on ICAM-1 and COX-2, the GO cells were treated with 10 ng/ml IL-1 β , with or without 1 μ M celastrol, for 24 h, following which the cells were collected and subjected to RT-qPCR analysis.

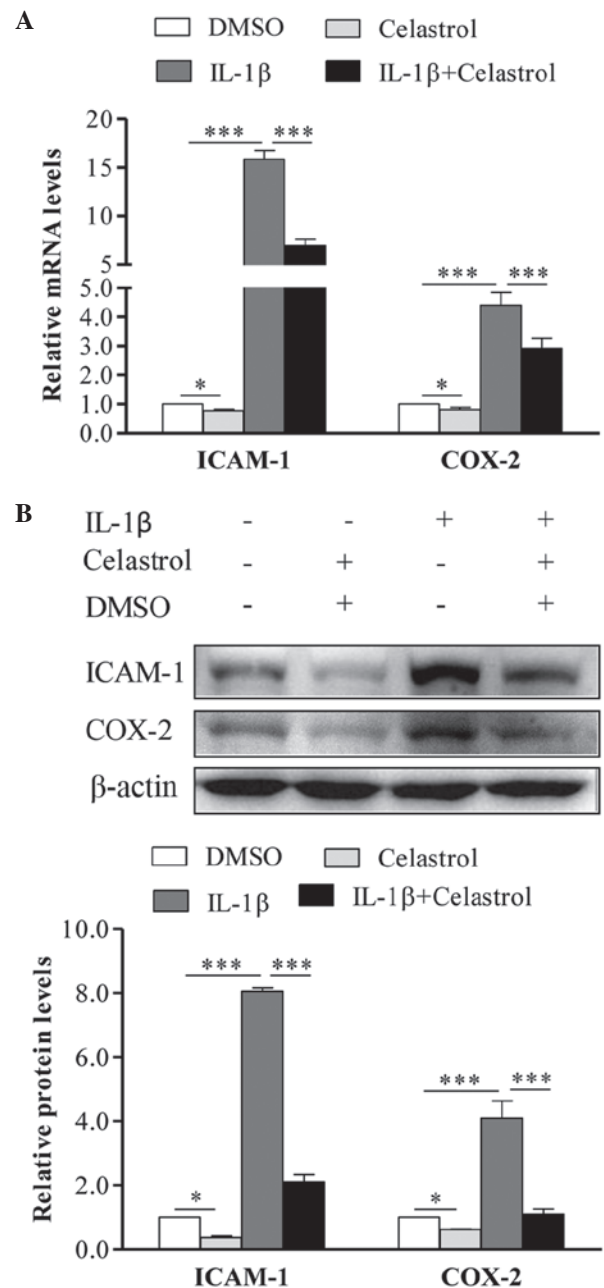


Figure 3. Celastrol suppresses the expression of IL-1 β -induced ICAM-1 and COX-2 in orbital fibroblasts. Orbital fibroblasts from patients with Graves' ophthalmopathy were stimulated with IL-1 β (10 ng/ml), with or without 1 μ M celastrol, for 24 h. (A) mRNA expression levels of ICAM-1 and COX-2 using reverse transcription-quantitative polymerase chain reaction analysis. (B) Protein expression levels of ICAM-1 and COX-2 were determined using western blot analysis. β -actin was used as an internal control. The graph shows representative data of expressed as the mean \pm standard error of the mean of three independent experiments. * P <0.05; *** P <0.01. IL-1 β , interleukin-1 β ; ICAM-1, intercellular adhesion molecule-1; COX-2, cyclooxygenase-2; DMSO, dimethyl sulfoxide.

As shown in Fig. 3A, in the IL-1 β -induced orbital fibroblasts, the mRNA expression levels of ICAM-1 and COX-2 were significantly increased, whereas treatment with celastrol almost completely reversed the IL-1 β -induced upregulation of ICAM-1 and COX-2. In addition, following treatment with IL-1 β , the protein expression levels of ICAM-1 and COX-2 were markedly enhanced, and this was also depressed by celastrol (Fig. 3B).

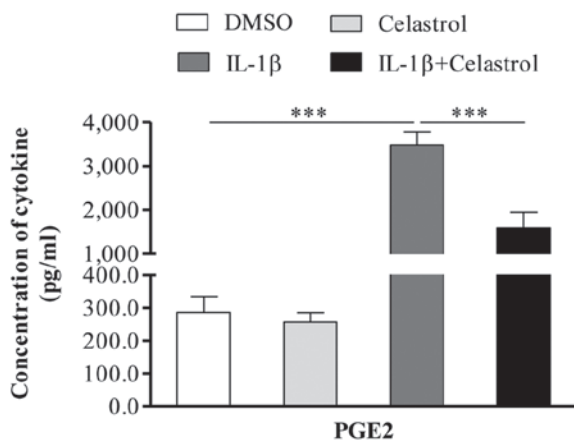


Figure 4. Celastrol suppresses the expression of IL-1 β -induced PGE2 in orbital fibroblasts. Orbital fibroblasts from patients with Graves' ophthalmopathy were stimulated with IL-1 β (10 ng/ml), with or without 1 μ M celastrol, for 24 h. Expression levels of PGE2 were determined using reverse transcription-quantitative polymerase chain reaction analysis. The figure shows representative data from three independent experiments. Data are expressed as the mean \pm standard error of the mean. ***P<0.01. IL-1 β , interleukin-1 β ; PGE2, prostaglandin E2; DMSO, dimethyl sulfoxide.

Effect of celastrol on IL-1 β -induced PGE2 in GO orbital fibroblasts. PGE2 is important in modulating the inflammatory process, and COX-2 is a key enzyme, which catalyzes the production of PGE2. It has been suggested that the increase in PGE2 may be attributed to the pathological inflammatory process of GO. As it was found that the IL-1 β -induced expression of COX-2 was depressed by celastrol, the present study evaluated the effect of celastrol on the IL-1 β -induced expression of PGE2. Following treatment of the GO orbital fibroblasts with 10 ng/ml IL-1 β , with or without 1 μ M celastrol for 24 h, the supernatants were analyzed using ELISA to detect the production of PGE2. As shown in Fig. 4, IL-1 β significantly induced the production of PGE2 in the orbital fibroblasts, whereas co-treatment with celastrol markedly attenuated the IL-1 β -induced expression of PGE2.

Effect of celastrol on the NF- κ B signaling pathway in GO orbital fibroblasts. The NF- κ B signaling pathway is important in regulating the production of several cytokines. In the cytoplasm, NF- κ B is arrested by I κ B, and the activation of IKK phosphorylates I κ B, thereby releasing NF- κ B, which translocates to the nucleus and activates the transcription of response genes (31). It has been demonstrated that celastrol is a potent inhibitor of NF- κ B, therefore, the present study examined whether celastrol exerts suppressive effects on IL-1 β -induced proinflammatory molecules through the inhibition of NF- κ B. As shown in Fig. 5A-C, following treatment with IL-1 β , the phosphorylation of I κ B α was significantly upregulated, whereas cotreatment with celastrol significantly suppressed the IL-1 β -induced phosphorylation of I κ B α . Pretreatment with the NF- κ B inhibitor, BAY-11-7082, almost completely inhibited the activation of NF- κ B induced by IL-1 β .

The effect of celastrol was also examined using an NF- κ B luciferase system in 293T cells. As shown in Fig. 5B, celastrol significantly inhibited IL-1 β -induced NF- κ B activation, in a dose-dependent manner.

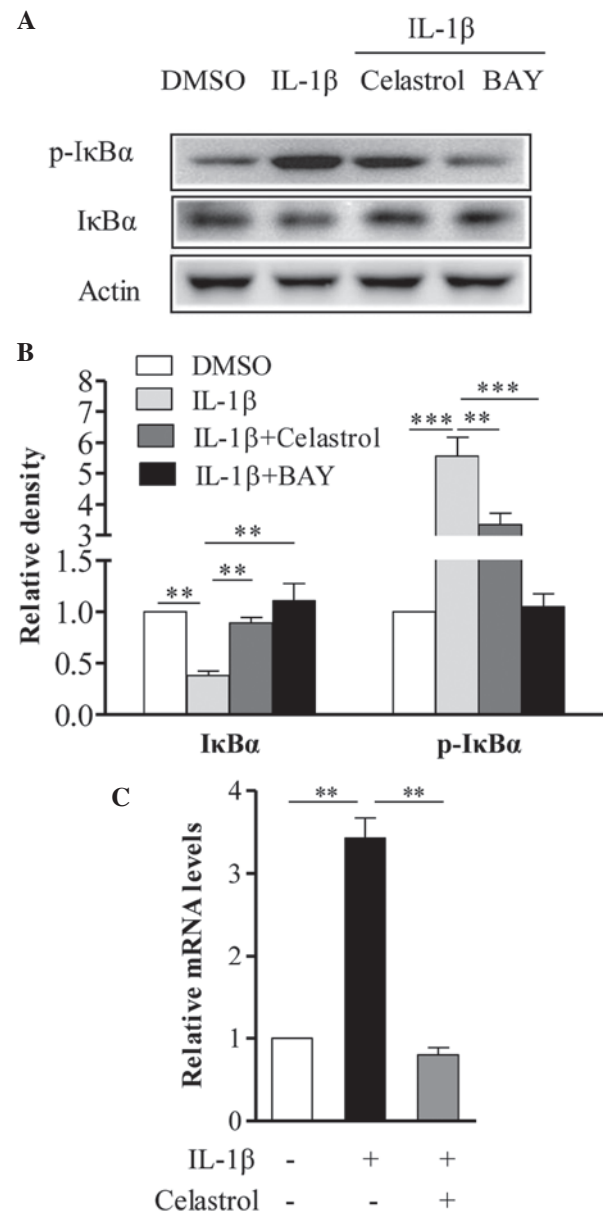


Figure 5. Celastrol suppresses the IL-1 β -induced expression and activation of the NF- κ B signaling pathway in orbital fibroblasts. (A) Orbital fibroblasts from patients with Graves' ophthalmopathy were stimulated with IL-1 β (10 ng/ml), with or without 1 μ M celastrol, for 24 h. Western blot analysis was performed to determine the phosphorylation of I κ B α . (B) Luciferase activity in 293T cells treated with celastrol. Cells were transfected with an NF- κ B luciferase reporter vector. After 24 h, the cells were stimulated with 10 ng/ml LPS with or without 1 μ M celastrol, for 24 h and then used for a luciferase assay. (C) Orbital fibroblasts were pretreated with BAY-11-7082 (2.5 μ M) for 30 min, then stimulated with 10 ng/ml LPS, with or without 1 μ M celastrol, for 24 h. Cells were lysed for western blot analysis to detect the expression of I κ B α and phosphorylation of I κ B α . Data is presented as the mean \pm standard error of the mean of three independent experiments. ***P<0.01; **P<0.05. IL-1 β , interleukin-1 β ; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; I κ B α , inhibitor of κ B α ; p-, phosphorylated; DMSO, dimethyl sulfoxide; BAY, BAY-11-7082.

Celastrol suppresses the induction of cytokines by IL-1 β in orbital fibroblasts through inhibition of the NF- κ B signaling pathway. To further determine whether the IL-1 β -induced stimulation of proinflammatory gene expression was mediated by the NF- κ B-dependent pathway, the present study pretreated GO cells with BAY-11-7082 (2.5 μ M) for 30 min,

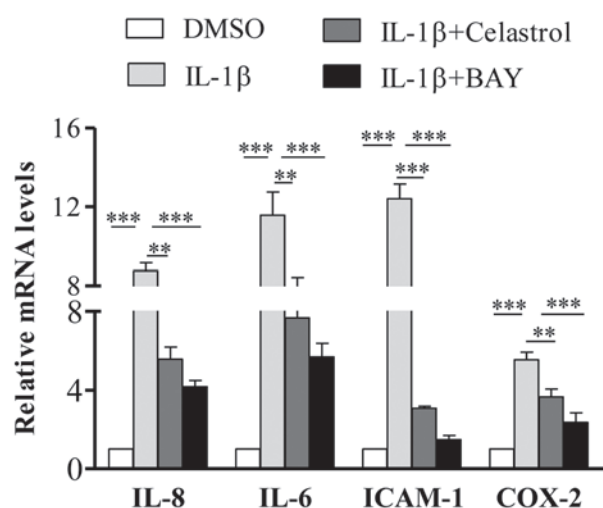


Figure 6. Celastrol suppresses cytokines induced by IL-1 β in orbital fibroblasts by inhibiting NF- κ B activity. Orbital fibroblasts from patients with Graves' ophthalmopathy were pre-incubated with BAY-11-7082 (2.5 μ M) for 30 min, following which IL-1 β and/or celastrol were added. Following incubation for 24 h, the cells were harvested and subjected to reverse transcription-quantitative polymerase chain reaction analysis. The figure shows representative data presented as the mean \pm standard error of the mean of three independent experiments. **P<0.01; ***P<0.001. IL, interleukin; ICAM-1, intercellular adhesion molecule-1; COX-2, cyclooxygenase-2; DMSO, dimethyl sulfoxide; BAY, BAY-11-7082.

following which IL-1 β and/or celastrol were added. Following incubation for 24 h, the cells were harvested and subjected to RT-qPCR analysis. The results showed that pre-incubation with BAY-11-7082 significantly decreased the IL-1 β -induced gene expression levels of IL-6, IL-8, ICAM-1 and COX-2 (Fig. 6), which confirmed activation of the NF- κ B pathway as the mechanism underlying the increased expression of these cytokines.

Discussion

Celastrol is generally used for the treatment of inflammatory and autoimmune diseases, however, the role of celastrol in the development of GO remains to be fully elucidated. In the present study, it was found that treatment with celastrol significantly attenuated inflammatory responses in IL-1 β -induced orbital fibroblasts from patients with GO through inhibiting the activation of NF- κ B. These results suggested that the use of celastrol may offer potential in the management of GO.

GO is an autoimmune disease, which is characterized by the infiltration of immune cells into the orbit and the production of excess glycosaminoglycans and inflammatory cytokines, which regulate the inflammatory response through recruiting and activating inflammatory cells. It has been suggested that cytokines are critical in the development of GO, as several cytokines have been detected in orbital tissues from patients with GO, including IL-1 β , IL-6, IL-8, COX-2 and ICAM-1 (12,32). It is well known that cytokines are produced predominantly by immune cells, and several have suggested that orbital fibroblasts are another important source of cytokines, which are critical in initiating and maintaining inflammation (33), with accumulating evidence suggesting that orbital fibroblasts are the autoimmune target and effector

cells in GO (34-36). IL-1 β is an important member of the IL-1 cytokine family, and mRNA expression levels of IL-1 β have been reported to be high in the orbital tissues of patients with GO (37). IL-1 β is involved in mediating the inflammatory response, and it has been reported that IL-1 β induces several mediators that have been correlated with the pathogenesis of GO, including IL-6 (38), IL-8 (39) and hyaluronic acid (40). In the present study, it was demonstrated that celastrol significantly suppressed the production of cytokines IL-6 and IL-8 in the orbital fibroblast induced by IL-1 β .

ICAM-1 was also induced by IL-1 β in the orbital fibroblasts, and the expression of ICAM-1 has been reported to be involved in the migration of lymphocytes to inflammatory sites in the orbit (41). The induction of COX-2 is considered to be critical to the inflammatory response in patients with GO. Orbital fibroblasts from the patients with GO treated by IL-1 β produced high levels of COX-2, and there is a positive correlation between the expression of COX-2 and the increasing severity of orbital disease (12). All these results suggested a possible association between the expression levels of ICAM-1 and COX2, and orbital inflammation in GO. Thus, the downregulation of these cytokines may result in decreased recruitment of leukocyte subsets into orbital fibroblasts. Pre-treatment of the orbital fibroblasts with celastrol had a potent inhibitory effect on the levels of IL-1 β -induced ICAM-1 and COX-2 in the IL-1 β -induced orbital fibroblasts. Together with the data described above, the results of the present study demonstrated that celastrol inhibited the production of the IL-6, IL-8, ICAM-1 and COX2 cytokines in orbital fibroblasts induced by IL-1 β , thereby suppressing the inflammatory response.

NF- κ B is a central transcription factor, which is well established as a regulator in mediating inflammatory and innate immune responses. NF- κ B may be activated by various factors, including the IL-1 cytokine (42). NF- κ B is important in regulating cell proliferation and cell survival. In the inactive state, NF- κ B is located in the cytoplasm, bound to the inhibitory protein, I κ B α . Following stimulation, the IKK complex is activated, which results in the phosphorylation and subsequent degradation of I κ B α , leading to the release of NF- κ B and its translocation to the nucleus, and activation of the transcription of target genes (43). Previous experiments have confirmed that the upregulation of COX-2 in GO is due to the activation of NF- κ B, and treatment with NF- κ B inhibitor almost completely suppresses IL-1 β -induced COX-2 in orbital fibroblast (32). Therefore, the effective inhibition of NF- κ B may be one of the therapeutic targets in GO.

Celastrol is a pharmacologically active compound, which possesses a broad range of biological activities and is generally used for the treatment of inflammatory and autoimmune diseases. Although several studies have demonstrated that celastrol offers therapeutic potential in a number of inflammatory-associated diseases *in vivo* and *in vitro* (17,18,44), its effects have not been investigated previously in GO. The application of celastrol has been controversial due to its toxicity. The present study showed that treatment with 1 μ M celastrol exerted no clear cytotoxic effects on the orbital fibroblast, and did not induce a significant level of apoptosis. This suggests that celastrol has realistic potential in clinical application. Celastrol is considered an inhibitor of NF- κ B, and several studies have demonstrated potent inhibitory effects on NF- κ B in various types of cell (45,46). Of note, in the

present study, celastrol was found to significantly suppress the production of cytokines induced by IL-1 β in orbital fibroblasts, and the levels of PGE2 in the IL-1 β -induced orbital fibroblasts was also inhibited by celastrol.

The results of the present study suggested that celastrol attenuated the IL-1 β -induced pro-inflammatory pathway in orbital fibroblasts from patients with GO, which was associated with the suppression of NF- κ B. The present study was the first, to the best of our knowledge, to evaluate the anti-inflammatory effects of celastrol on orbital fibroblasts in patients with GO, and the results suggested that celastrol may be efficient in the treatment of GO, in terms of attenuating the inflammatory process.

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References

- Prabhakar BS, Bahn RS and Smith TJ: Current perspective on the pathogenesis of Graves' disease and ophthalmopathy. *Endocr Rev* 24: 802-835, 2003.
- Bahn RS: Graves' ophthalmopathy. *N Engl J Med* 362: 726-738, 2010.
- Garrity JA and Bahn RS: Pathogenesis of graves ophthalmopathy: Implications for prediction, prevention, and treatment. *Am J Ophthalmol* 142: 147-153, 2006.
- Wiersinga WM and Bartalena L: Epidemiology and prevention of Graves' ophthalmopathy. *Thyroid* 12: 855-860, 2002.
- Ajjan RA and Weetman AP: New understanding of the role of cytokines in the pathogenesis of Graves' ophthalmopathy. *J Endocrinol Invest* 27: 237-245, 2004.
- Kazim M, Goldberg RA and Smith TJ: Insights into the pathogenesis of thyroid-associated orbitopathy: Evolving rationale for therapy. *Arch Ophthalmol* 120: 380-386, 2002.
- Smith TJ: Orbital fibroblasts exhibit a novel pattern of responses to proinflammatory cytokines: Potential basis for the pathogenesis of the thyroid-associated ophthalmopathy. *Thyroid* 12: 197-203, 2002.
- Lee WM, Paik JS, Cho WK, Oh EH, Lee SB and Yang SW: Rapamycin enhances TNF- α -induced secretion of IL-6 and IL-8 through suppressing PDCD4 degradation in orbital fibroblasts. *Curr Eye Res* 38: 699-706, 2013.
- Yoon JS, Chae MK, Lee SY and Lee EJ: Anti-inflammatory effect of quercetin in a whole orbital tissue culture of Graves' orbitopathy. *Br J Ophthalmol* 96: 1117-1121, 2012.
- Heufelder AE and Bahn RS: Elevated expression in situ of selectin and immunoglobulin superfamily type adhesion molecules in retroocular connective tissues from patients with Graves' ophthalmopathy. *Clin Exp Immunol* 91: 381-389, 1993.
- Kahaly G, Hansen C, Felke B and Dienes HP: Immunohistochemical staining of retrobulbar adipose tissue in Graves' ophthalmopathy. *Clin Immunol Immunopathol* 73: 53-62, 1994.
- Konuk EB, Konuk O, Misirliglu M, Menevse A and Unal M: Expression of cyclooxygenase-2 in orbital fibroadipose connective tissues of Graves' ophthalmopathy patients. *Eur J Endocrinol* 155: 681-685, 2006.
- Wang HS, Cao HJ, Winn VD, Rezanka LJ, Frobert Y, Evans CH, Sciaky D, Young DA and Smith TJ: Leukoregulin induction of prostaglandin-endoperoxide H synthase-2 in human orbital fibroblasts. An in vitro model for connective tissue inflammation. *J Biol Chem* 271: 22718-22728, 1996.
- Bartalena L, Pinchera A and Marcocci C: Management of Graves' ophthalmopathy: Reality and perspectives. *Endocr Rev* 21: 168-199, 2000.
- Kannaiyan R, Shanmugam MK and Sethi G: Molecular targets of celastrol derived from Thunder of God Vine: Potential role in the treatment of inflammatory disorders and cancer. *Cancer Lett* 303: 9-20, 2011.
- Salminen A, Lehtonen M, Paimela T and Kaarniranta K: Celastrol: Molecular targets of Thunder God Vine. *Biochem Biophys Res Commun* 394: 439-442, 2010.
- Cascão R, Vidal B, Raquel H, Neves-Costa A, Figueiredo N, Gupta V, Fonseca JE and Moita LF: Effective treatment of rat adjuvant-induced arthritis by celastrol. *Autoimmun Rev* 11: 856-862, 2012.
- Li H, Zhang YY, Tan HW, Jia YF and Li D: Therapeutic effect of tripterine on adjuvant arthritis in rats. *J Ethnopharmacol* 118: 479-484, 2008.
- Kim DY, Park JW, Jeoung D and Ro JY: Celastrol suppresses allergen-induced airway inflammation in a mouse allergic asthma model. *Eur J Pharmacol* 612: 98-105, 2009.
- Li H, Zhang YY, Huang XY, Sun YN, Jia YF and Li D: Beneficial effect of tripterine on systemic lupus erythematosus induced by active chromatin in BALB/c mice. *Eur J Pharmacol* 512: 231-237, 2005.
- Kim DH, Shin EK, Kim YH, Lee BW, Jun JG, Park JH and Kim JK: Suppression of inflammatory responses by celastrol, a quinone methide triterpenoid isolated from *Celastrus regelii*. *Eur J Clin Invest* 39: 819-827, 2009.
- Sen T, Dutta A and Chatterjee A: Epigallocatechin-3-gallate (EGCG) downregulates gelatinase-B (MMP-9) by involvement of FAK/ERK/NF κ B and AP-1 in the human breast cancer cell line MDA-MB-231. *Anticancer Drugs* 21: 632-644, 2010.
- Ghosh S, May MJ and Kopp EB: NF- κ B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 16: 225-260, 1998.
- Lee JH, Koo TH, Yoon H, Jung HS, Jin HZ, Lee K, Hong YS and Lee JJ: Inhibition of NF- κ B activation through targeting I κ B kinase by celastrol, a quinone methide triterpenoid. *Biochem Pharmacol* 72: 1311-1321, 2006.
- Sethi G, Ahn KS, Pandey MK and Aggarwal BB: Celastrol, a novel triterpene, potentiates TNF-induced apoptosis and suppresses invasion of tumor cells by inhibiting NF- κ B-regulated gene products and TAK1-mediated NF- κ B activation. *Blood* 109: 2727-2735, 2007.
- Yiu WH, Wong DW, Chan LY, Leung JC, Chan KW, Lan HY, Lai KN and Tang SC: Tissue kallikrein mediates pro-inflammatory pathways and activation of protease-activated receptor-4 in proximal tubular epithelial cells. *PLoS One* 9: e88894, 2014.
- Liang C, Du W, Dong Q, Liu X, Li W, Wang Y and Gao G: Expression levels and genetic polymorphisms of interleukin-2 and interleukin-10 as biomarkers of Graves' disease. *Exp Ther Med* 9: 925-930, 2015.
- Zhao LQ, Wei RL, Cheng JW, Cai JP and Li Y: The expression of intercellular adhesion molecule-1 induced by CD40-CD40 L ligand signaling in orbital fibroblasts in patients with Graves' ophthalmopathy. *Invest Ophthalmol Vis Sci* 51: 4652-4660, 2010.
- Choi YH, Back KO, Kim HJ, Lee SY and Kook KH: Pirfenidone attenuates IL-1 β -induced COX-2 and PGE2 production in orbital fibroblasts through suppression of NF- κ B activity. *Exp Eye Res* 113: 1-8, 2013.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Chen LF and Greene WC: Shaping the nuclear action of NF- κ B. *Nat Rev Mol Cell Biol* 5: 392-401, 2004.
- Yoon JS, Lee HJ, Choi SH, Chang EJ, Lee SY and Lee EJ: Quercetin inhibits IL-1 β -induced inflammation, hyaluronan production and adipogenesis in orbital fibroblasts from Graves' orbitopathy. *PLoS One* 6: e26261, 2011.
- Smith TJ: Unique properties of orbital connective tissue underlie its involvement in Graves' disease. *Minerva Endocrinol* 28: 213-222, 2003.
- Smith TJ: Novel aspects of orbital fibroblast pathology. *J Endocrinol Invest* 27: 246-253, 2004.
- Smith RS, Smith TJ, Blieden TM and Phipps RP: Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation. *Am J Pathol* 151: 317-322, 1997.
- Cao HJ, Wang HS, Zhang Y, Lin HY, Phipps RP and Smith TJ: Activation of human orbital fibroblasts through CD40 engagement results in a dramatic induction of hyaluronan synthesis and prostaglandin endoperoxide H synthase-2 expression. Insights into potential pathogenic mechanisms of thyroid-associated ophthalmopathy. *J Biol Chem* 273: 29615-29625, 1998.
- Wakelkamp IM, Bakker O, Baldeschi L, Wiersinga WM and Prummel MF: TSH-R expression and cytokine profile in orbital tissue of active vs. Inactive Graves' ophthalmopathy patients. *Clin Endocrinol (Oxf)* 58: 280-287, 2003.

38. Chen B, Tsui S and Smith TJ: IL-1 beta induces IL-6 expression in human orbital fibroblasts: Identification of an anatomic-site specific phenotypic attribute relevant to thyroid-associated ophthalmopathy. *J Immunol* 175: 1310-1319, 2005.
39. Hwang CJ, Afifiyan N, Sand D, Naik V, Said J, Pollock SJ, Chen B, Phipps RP, Goldberg RA, Smith TJ and Douglas RS: Orbital fibroblasts from patients with thyroid-associated ophthalmopathy overexpress CD40: CD154 hyperinduces IL-6, IL-8 and MCP-1. *Invest Ophthalmol Vis Sci* 50: 2262-2268, 2009.
40. Kaback LA and Smith TJ: Expression of hyaluronan synthase messenger ribonucleic acids and their induction by interleukin-1beta in human orbital fibroblasts: Potential insight into the molecular pathogenesis of thyroid-associated ophthalmopathy. *J Clin Endocrinol Metab* 84: 4079-4084, 1999.
41. Sikorski EE, Hallmann R, Berg EL and Butcher EC: The Peyer's patch high endothelial receptor for lymphocytes, the mucosal vascular addressin, is induced on a murine endothelial cell line by tumor necrosis factor-alpha and IL-1. *J Immunol* 151: 5239-5250, 1993.
42. Lie PP, Cheng CY and Mruk DD: The biology of interleukin-1: Emerging concepts in the regulation of the actin cytoskeleton and cell junction dynamics. *Cell Mol Life Sci* 69: 487-500, 2012.
43. Tak PP and Firestein GS: NF-kappaB: A key role in inflammatory diseases. *J Clin Invest* 107: 7-11, 2001.
44. Kiaei M, Kipiani K, Petri S, Chen J, Calingasan NY and Beal MF: Celastrol blocks neuronal cell death and extends life in transgenic mouse model of amyotrophic lateral sclerosis. *Neuro-degener Dis* 2: 246-254, 2005.
45. He D, Xu Q, Yan M, Zhang P, Zhou X, Zhang Z, Duan W, Zhong L, Ye D and Chen W: The NF-kappa B inhibitor, celastrol, could enhance the anti-cancer effect of gambogic acid on oral squamous cell carcinoma. *BMC Cancer* 9: 343, 2009.
46. Zhou LL, Lin ZX, Fung KP, Cheng CH, Che CT, Zhao M, Wu SH and Zuo Z: Celastrol-induced apoptosis in human HaCaT keratinocytes involves the inhibition of NF- κ B activity. *Eur J Pharmacol* 670: 399-408, 2011.