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Inhibition of NF-κB activation through targeting IκB kinase by celastrol, a quinone methide triterpenoid

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Abbreviations:
NF-κB, nuclear factor κB
AP-1, activator protein-1
LPS, lipopolysaccharide
TNF-α, tumor necrosis factor-α
PMA, phorbol myristyl acetate
MEKK-1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1
IKK, IκB kinase
EMSA, electrophoretic mobility shift assay
COX-2, cyclooxygenase-2
PGE_2, prostaglandin E_2
iNOS, inducible nitric oxide synthase
NO, nitric oxide
MPO, myeloperoxidase
DTT, dithiothreitol

ABSTRACT

Celastrol, a quinone methide triterpenoid, was isolated as an inhibitor of NF-κB from *Celastrus orbiculatus*. This compound dose-dependently inhibited a variety of stimuli-induced NF-κB-regulated gene expression and the DNA-binding of NF-κB in different cell lines without affecting DNA-binding activity of AP-1. Preincubation of celastrol completely blocked the LPS-, TNF-α-, or PMA-induced degradation and phosphorylation of IκBα. Importantly, celastrol inhibited IKK activity and the constitutively active IKKβ activity in a dose-dependent manner without either affecting the NF-κB activation induced by RelA over-expression or directly suppressing the DNA-binding of activated NF-κB. However, mutation of cysteine 179 in the activation loop of IKKβ abolished sensitivity towards to celastrol, suggesting that celastrol suppressed the NF-κB activation by targeting cysteine 179 in the IKK. To verify that celastrol is a NF-κB inhibitor, we investigated its effect on some NF-κB target genes expressions. Celastrol prevented not only LPS-induced mRNA expression of iNOS and TNF-α, but also TNF-α-induced Bfl-1/A1 expression, a prosurvival Bcl-2 homologue. Consistent with these results, celastrol significantly suppressed the production of NO and TNF-α in LPS-stimulated RAW264.7 cells, and increased the cytotoxicity of TNF-α in HT-1080 cells. We also demonstrated that celastrol showed anti-inflammatory and anti-tumor activities in animal models. Taken together, this study extends our understanding on the molecular mechanisms underlying the anti-inflammatory and anti-cancer activities of celastrol and celastrol-containing medicinal plant, which would be a valuable candidate for the intervention of NF-κB-dependent pathological conditions.

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1. Introduction

NF-κB regulates the transcription of a large number of genes, particularly those involved in immune, inflammatory, and antiapoptotic responses [1]. Inappropriate regulation of NF-κB is directly involved in a wide range of human disorders, including a variety of cancers, neurodegenerative diseases, ataxiatelangiectasia, arthritis, asthma, inflammatory bowel disease, and numerous other inflammatory conditions [2]. In most cell types, the dimeric transcription factor NF-κB is trapped in the cytoplasm by binding to its inhibitor protein IκBs. IκBs retain NF-κB in the cytoplasm by masking the nuclear localization sequence contained in the Rel homology domain [3,4]. The latent NF-κB is activated by a variety of cellular stimuli, which trigger site-specific phosphorylation of IκBα by a IκB kinase complex [5,6]. The phosphorylated IκBα becomes rapidly ubiquitinated and degraded by the proteasome complex [7,8]. Following IκBα degradation, the NF-κB is translocated to the nucleus, where it activates the transcription of target genes.

NF-κB regulates the transcription of various inflammatory cytokines, such as interleukin-1, -2, -6, -8 and TNF-α, as well as genes encoding COX-2, iNOS, immunoreceptors, cell adhesion molecules, hematopoietic growth factors, and growth factor receptors [9]. In addition to regulating the expression of genes important for immune and inflammatory responses, NF-κB also controls the transcription of genes that confer resistance to death-inducing signals. Target genes include those encoding the caspase inhibitors, c-IAP1s and anti-apoptotic regulators, such as Bfl-1/A1 and GADD45β [10]. Interestingly, recent findings show that activation of NF-κB may function as a tumor promoter in inflammation-associated cancer, suggesting that NF-κB is a key player linking between chronic inflammation and cancer [11,12].

NF-κB and the signaling pathways that regulate its activity have become a focal point for intense drug discovery and development efforts [13]. Well-known anti-inflammatory substances such as glucocorticoids or nonsteroidal anti-inflammatory drugs (NSAIDs) exert, at least a part of their effects, by inhibiting NF-κB activity [14–16]. Several anti-inflammatory medicinal plants also contain structurally diverse compounds that inhibit NF-κB activation [17–19]. In our search for inhibitors of NF-κB activation from natural products, we identified celastrol, a quinone methide triterpenoid as a major component of active principles in the methanol extract of the roots of *Celastrus orbiculatus* [20], which has been used as a treatment for rheumatoid arthritis and bacterial infection in folk medicine [21].

In this study, we investigated the effect of celastrol on the NF-κB activation and anti-inflammatory and anti-tumor activities. Our results show that celastrol suppressed the NF-κB activation by inhibiting IKK activity, possibly through directly targeting cysteine 179 in the activation loop of IKK. Celastrol prevented not only the expression of iNOS, TNF-α, and Bfl-1/A1, but also the production of NO and TNF-α in LPS-stimulated RAW264.7 cells and increased the cytotoxicity of TNF-α in HT-1080 cells. Furthermore, celastrol showed anti-inflammatory and anti-tumor activities in animal models. Our results suggest that celastrol is a valuable compound for modulation of NF-κB-dependent pathological conditions and support pharmacological basis that the *Celastraceae* plants have been used as a traditional herbal medicine for the treatments of inflammation and cancer.

2. Materials and methods

2.1. Cell culture and reagents

Human Jurkat T leukemia cells and monocyte U937 cells were maintained in RPMI 1640 medium. RAW264.7 cells, HeLa cells, human 293 embryonic kidney cells and HT-1080 cells were maintained in Dulbecco's modified Eagle's medium. MDA-MB-435 cells were the generous gift of Dr. Danny R. Welch (University of Alabama at Birmingham, Birmingham, AL, USA) and maintained in DME-F12 medium. All media were supplemented with penicillin (100 units/ml)–streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA, USA) and 10% heat-inactivated fetal bovine serum (Invitrogen). All cells were grown in an incubator at 37 °C and 5% CO_2. TNF-α was obtained from R&D Systems (Minneapolis, MN, USA), and PMA and LPS from Sigma–Aldrich (St. Louis, MO, USA). Celastrol was isolated from *C. orbiculatus* and confirmed its structure (supplementary data 2) in comparison with the previous report and its purity is more than 98% in HPLC analysis [22].

2.2. Plasmids, transfections and luciferase reporter gene assay

A pNFκB-Luc plasmid for NF-κB luciferase reporter assay was obtained from Stratagene (La Jolla, CA, USA). Expression vectors for IKKα and IKKβ, IKKβ (SS/EE) and IKKβ (SS/EE/C179A), and RelA and MEKK-1 were kindly provided from Dr. M. Karin (University of California San Diego, USA), Dr. C.M. Crew (Yale University, USA), and Dr. Mira Jung (Georgetown University, DC, USA), respectively. A cDNA for Bfl-1/A1 was kindly provided from Dr. Sukil Hong (Korea Cancer Research Center, Seoul, Korea). Transfections were performed using Lipofectamine plus reagent according to the instructions of manufacturer (Invitrogen). Luciferase assay was performed using Dual-Luciferase Assay System according to the instructions of the manufacturer (Promega, Madison, WI, USA). Luciferase activity was determined in Microlumat Plus luminometer (EG&G Berthold, Bad Wildbad, Germany) by injecting 100 μl of assay buffer containing substrate and measuring light emission for 10 s. Results are expressed as means of the ratio of between firefly luciferase activity and *Renilla* luciferase activity.

2.3. Electrophoretic mobility shift assay (EMSA)

Thirty minutes prior to stimulation with TNF-α, LPS, or PMA, cells were preincubated with the indicated concentrations of celastrol at 37 °C. In following, cells were stimulated with TNF-α (20 ng/ml), PMA (50 ng/ml), or LPS (10 μg/ml) and harvested by centrifugation and washed twice with cold phosphate-buffered saline. EMSA was performed as described previously [19]. Briefly, 2×10^6 cells were resuspended in 400 μl of cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride,

2.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin) by flicking tube. The cells were allowed to swell on ice for 15 min, and then vortex for 20 s. The homogenate was centrifuged for 30 s in a Microfuge at 4 °C. The pellet was resuspended in 50 ml of ice-cold extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 2.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin), and incubated on ice for 20 min with intermittent mixing. Cellular debris was removed by centrifugation for 5 min at 13,000 rpm at 4 °C. The supernatant (nuclear extract) was either used immediately or stored at −70 °C for later use. Electrophoretic mobility shift assay was performed using gel shift assay system (Promega), according to the instructions of manufacturer. A double-stranded oligonucleotide for NF-κB (Promega) or AP-1 (Promega) was end-labeled with [γ-^{32}P] ATP and purified with a G-25 spin column (Boehringer Mannheim, Mannheim, Germany). Nuclear extracts (10 μg) were incubated for 20 min at room temperature with a gel shift binding buffer [5% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl, pH 7.5, 50 μg/ml poly(dI–dC)] and ^{32}P-labeled oligonucleotide. The DNA–protein complex formed was separated on 4% native polyacrylamide gels. The gel was transferred to Whatman 3 MM paper, dried, and exposed to X-ray film at −70 °C with an intensifying screen. The specificity of binding was also examined by competition with an excess of unlabelled oligonucleotide.

2.4. Western blotting

Proteins were extracted from cells in ice-cold lysis buffer (50 mM Tris–HCl pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin). Fifty micrograms of protein per lane was separated by SDS-polyacrylamide gels and followed by transferring to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk, and then incubated with the antibodies for IκBα or phospho-specific IκBα (New England Biolabs Inc., Beverly, MA, USA). After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Pharmacia Biotec, Buckinghamshire, UK).

2.5. Northern blot analysis

Total RNAs were isolated using RNeasy Mini Kits according to the instructions of the manufacturer (Qiagen, Valencia, CA, USA). Ten micrograms of total RNA were resolved on 1% agarose-formaldehyde gel and transferred to a nylon membrane by capillary action. Membranes were probed and washed according to the instructions of the manufacturer (Boehringer Mannheim). ^{32}P-labelled probes for Bfl-1/A1, iNOS, and TNF-α were generated by the random priming method using Rediprime II (Amersham Pharmacia Biotec) and 50 μCi [α-^{32}P]dCTP (3000 Ci/mmol; NEN). Unincorporated nucleotides were removed by purification through a G-25 spin column. The results were visualized by autoradiography. Quantitation was determined by a densitometry.

2.6. Kinase assays

Human 293 cells grown in 100 mm plates were transfected with expression vectors for IKKα, IKKβ, IKKβ (SS/EE), or IKKβ (SS/EE/C179A) and incubated for 48 h. The cells transfected with IKKα or IKKβ were stimulated with TNF-α (20 ng/ml) for 20 min, and then washed three times with ice-cold PBS containing 1 mM Na_3VO_4 and 5 mM EDTA. The cells transfected with IKKβ (SS/EE) or IKKβ (SS/EE/C179A) washed three times with ice-cold PBS without stimulation. Cell lysates prepared in lysis buffer (20 mM Tris–HCl, 0.5 M NaCl, 0.25% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM 4-nitrophenylphosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin, and 1 mM DTT) were incubated with antibodies on ice for 2 h. Protein A- or protein G-conjugated agarose beads were then added and incubated for additional 2 h at 4 °C. To evaluate the effect of celastrol on the activation of IKK in cells, HeLa cells were stimulated with TNF-α (20 ng/ml) for 20 min with or without various concentrations of celastrol, and the endogenous IKK complex was prepared with anti-IKKβ antibody for kinase assay. Kinase assays were performed by incubating the immune complexes in kinase reaction buffer (20 mM HEPES pH 7.7, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP, 10 mM β-glycerophosphate, 10 mM NaF, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin, and 1 mM DTT) with 5 μCi of [γ-^{32}P]ATP and bacterially expressed GST-IκBα in a reaction volume of 20 μl for 30 min at 30 °C. Samples were analyzed by 12.5% SDS-PAGE, autoradiography, and Western blotting. Anti-IKKα, anti-IKKβ, and anti-HA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phosphorylation of GST-IκBα was quantitated by a densitometry.

2.7. Measurement of NO and TNF-α and cell viability

RAW 264.7 cells grown on 100 mm culture dish were harvested and seeded in 96-well plates at 2×10^5 cells/well for NO or at 2×10^4 cells/well for TNF-α. The plates were pretreated with various concentrations of celastrol for 30 min and then incubated for 24 h with or without 1 μg/ml of LPS. Nitrite concentration in the culture supernatant was measured by the Griess reaction [23]. The amount of TNF-α in the culture supernatant was measured using a TNF-α ELISA kit (Genzyme, Cambridge, MA, USA). Cell viability was measured by a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma–Aldrich). Briefly, untreated cells or treated cells with celastrol and/or TNF-α in a 96-well plate were incubated for 48 h followed by the addition of MTT to the cells. Optical densities were determined on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.8. Anti-inflammatory activity of celastrol in vivo

To assess anti-inflammatory activity of celastrol, we used an air-pouch model of inflammation as previously described [24,25]. In brief, air cavities were produced by subcutaneous injection of 5 ml of sterile air into the intracapsular area of the

back. An additional 3 ml of air was injected into the air cavity 3 days after first injection. Six days after the initial air injection, 1 ml of a 1% solution of carrageenan dissolved in saline was injected into the pouch to produce an inflammatory response. Celastrol was administered intraperitoneally prior to carrageenan injection. Four hours after the carrageenan injection, the inflammatory exudates were harvested. Neutrophil accumulation in the air pouch was assessed by measurement of myeloperoxidase (MPO) activity in pouch inflammatory exudates. In brief, 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer pH 6.0 was used to solubilize MPO from neutrophils. Cell debris was removed by centrifugation (2500 × *g*, 15 min, 4 °C). Ten microliters of supernatant was added to 190 μl of 50 mM potassium phosphate buffer pH 6.0 containing 0.5 mM *o*-dianisidine and 0.001% hydrogen peroxide. Absorbance at 450 nm was measured for 2 min and MPO activity was normalized with commercially available human myeloperoxidase (Sigma). PGE_2 and TNF-α were determined in the pouch exudates as previously described [25]. Data were analyzed using ANOVA (analysis of variance).

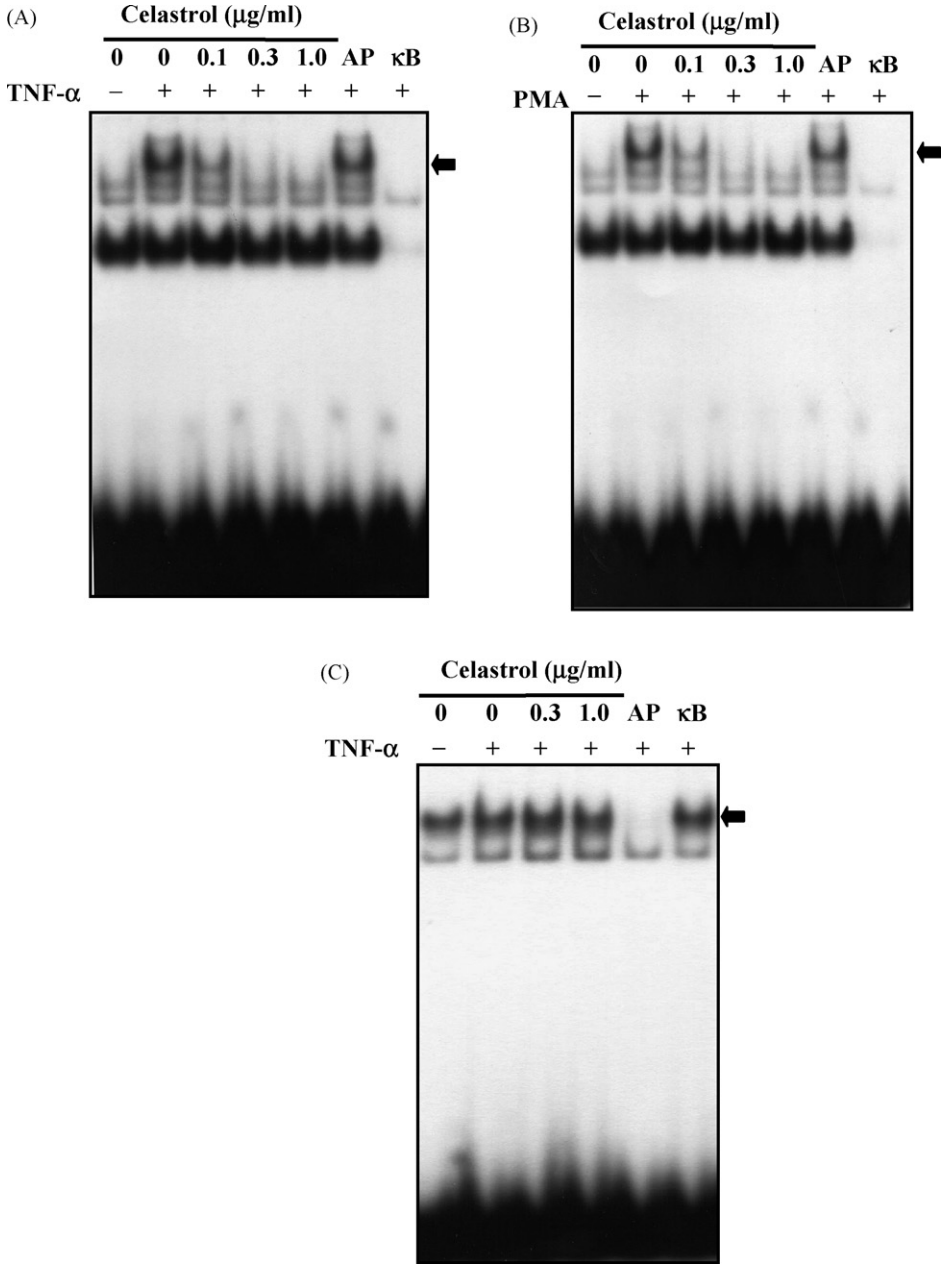

Fig. 1 – . Celastrol inhibits NF-κB activation by different stimuli. (A and B) Jurkat T cells were preincubated for 30 min with the indicated concentrations of celastrol and followed by the stimulation with 20 ng/ml of TNF-α (A) or 50 ng/ml of PMA (B) for 90 min. Subsequently nuclear extracts were prepared and tested for DNA-binding of activated NF-κB by EMSA. (C) Nuclear extracts prepared in A were tested for DNA-binding of activated AP-1 by EMSA. In lane AP; a 100-fold excess of unlabeled AP-1 consensus oligonucleotide was added to the reaction mixture. In lane κB; a 100-fold excess of unlabeled NF-κB oligonucleotide was added to the reaction mixture. The arrow indicates the location of the DNA–NF-κB or DNA–AP-1 complex.

2.9. Anti-tumor activity of celastrol in vivo

MDA-MB-435 cells (10^6) were injected into the subaxillary mammary fat pads of 4–6-week-old female athymic nude mice (Charles River Laboratory, Wilmington, MA, USA) as described [26]. All experiments were performed according to ethical guidelines for the use of laboratory animals as adopted by the United States National Institute of Health. Food and water were provided *ad libitum*. Each xenograft was monitored weekly by externally measuring tumors in two dimensions using a caliper. Tumor volume (V) was determined by the following equation: $V = (L \times W^2) \times 0.5$, where L is the length and W is the width of a xenograft. When tumors reached a volume of approximately 100 mm^3, the mice were randomly divided into three experimental groups of eight animals each, and received the following treatment every other day via intraperitoneal (i.p.) injection: group 1 (control), vehicle solution; group 2, celastrol at a dose of 10 mg/kg per animal; group 3, celastrol at a dose of 30 mg/kg per animal. The treatment was continued for 7 weeks.

3. Results

3.1. Celastrol inhibits NF-κB activation by different stimuli

In our effort to identify NF-κB inhibitors from natural resources, celastrol was isolated from *C. orbiculatus* [20]. This compound dose-dependently suppressed the LPS-, PMA-, and TNF-α-induced expression of NF-κB reporter gene. In this study, we investigated the inhibitory effect of celastrol on the NF-κB activation. Two cell lines, human lymphoma Jurkat, and human monocyte U937, were preincubated with various concentrations of celastrol for 30 min prior to stimulation. Jurkat cells were stimulated for 30 min with TNF-α or PMA, and U937 cells for 30 min with LPS. After the stimulation, nuclear extracts were prepared and DNA-binding activity of NF-κB in the nuclear extracts was measured by electrophoretic mobility shift assays (Fig. 1A and B). Jurkat cells stimulated with TNF-α or PMA strongly induced DNA-binding activity of NF-κB. However, pretreatment of celastrol dose-dependently inhibited DNA-binding activity of NF-κB induced by TNF-α or PMA. Similar inhibitory effect of celastrol on NF-κB activation by LPS was obtained from U937 cells (data not shown). To examine if the effect of celastrol on the NF-κB activation is specific, we measured DNA-binding activity of AP-1 using the same nuclear extracts as in Fig. 1A. Celastrol did not significantly affect DNA-binding activity of AP-1 (Fig. 1C).

3.2. Celastrol inhibits NF-κB reporter gene expression induced by over-expression of IKKβ, or MEKK-1 but not by over-expression of RelA

To further investigate the possible target of celastrol during NF-κB activation, we analyzed the effect of celastrol on the NF-κB reporter gene expression induced by over-expression of MEKK-1, IKKβ, or RelA. HeLa cells were transfected with a NF-κB reporter gene alone or in combination with expression vectors for MEKK-1, IKKβ, or RelA (Fig. 2). The TNF-α-induced NF-κB activation was efficiently impaired in the presence of

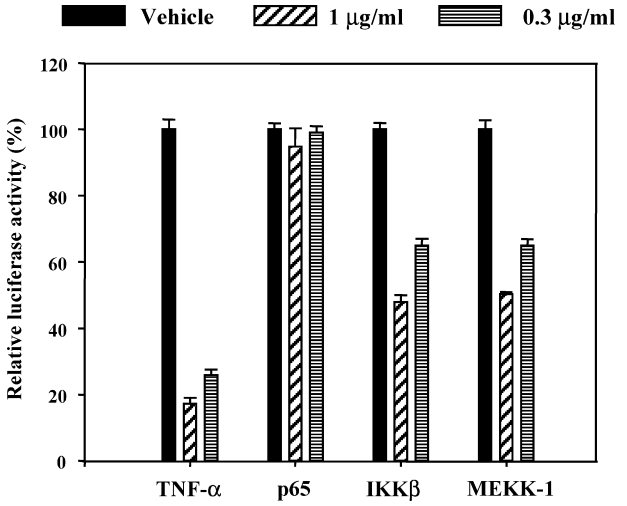

Fig. 2 – Effect of celastrol on the NF-κB activation induced by the over-expression of RelA, IKKβ, or MEKK-1. HeLa cells were transiently transfected with a NF-κB-dependent reporter gene together with expression vectors encoding RelA, IKKβ, or MEKK-1. The cotransfected cells were subsequently grown for 24 h and for another 12 h in the presence of the indicated concentrations of celastrol. As a control, HeLa cells transfected with a NF-κB-dependent reporter gene were stimulated with TNF-α (1 μg/ml) for 12 h in the presence of the indicated concentrations of celastrol. The luciferase activity was determined as described in Section 2. Mean values from three independent experiments performed in triplicated are shown; bar indicate the S.D.s.

celastrol. Similarly, NF-κB-dependent luciferase expression induced by over-expression of MEKK-1 and IKKβ was significantly affected by celastrol. However, NF-κB-dependent luciferase expression by RelA over-expression was not affected by celastrol. Taken together, these results suggest that celastrol could suppress one or more common steps during NF-κB activation prior to nuclear translocation, possibly by inhibiting IKK kinase activity.

3.3. Celastrol inhibits the degradation and phosphorylation of IκBα

Since degradation of IκB proteins is an essential step for NF-κB activation by various stimuli, we examined the effect of celastrol on the TNF-α- or PMA-induced IκBα degradation (Fig. 3A and B). Jurkat cells were pretreated with 1 μg/ml of celastrol for 30 min, and subsequently stimulated with TNF-α or PMA for the indicated times. Total cell extracts were analyzed the presence of IκBα with Western blots. IκBα was completely degraded in 30 min after stimulation with TNF-α or PMA. Preincubation of celastrol, however, completely prevented the TNF-α- or PMA-induced IκBα degradation. Similarly, celastrol prevented the LPS-induced IκBα degradation in U937 cells (data not shown). Furthermore, determination of IκBα phosphorylation by Western blot using a phospho-specific IκBα antibody revealed the significant inhibitory effect on the TNF-α -induced IκBα phosphorylation in the presence

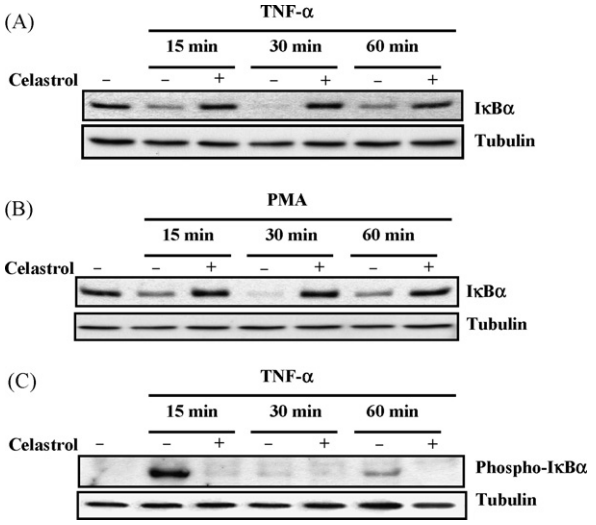

Fig. 3 – Effect of celastrol on IκBα degradation and phosphorylation induced by TNF-α and PMA. Jurkat T cells were pretreated for 30 min with 1 μg/ml of celastrol prior to stimulation with 20 ng/ml of TNF-α (A and C), or 50 ng/ml of PMA (B). Cells were harvested at the indicated time points and total cell extracts were prepared. IκBα protein (A and B) and phospho-IκBα protein (C) were detected by Western blot analysis as described in Section 2. The bottom represents α-tubulin to show the equal loading of cell lysates.

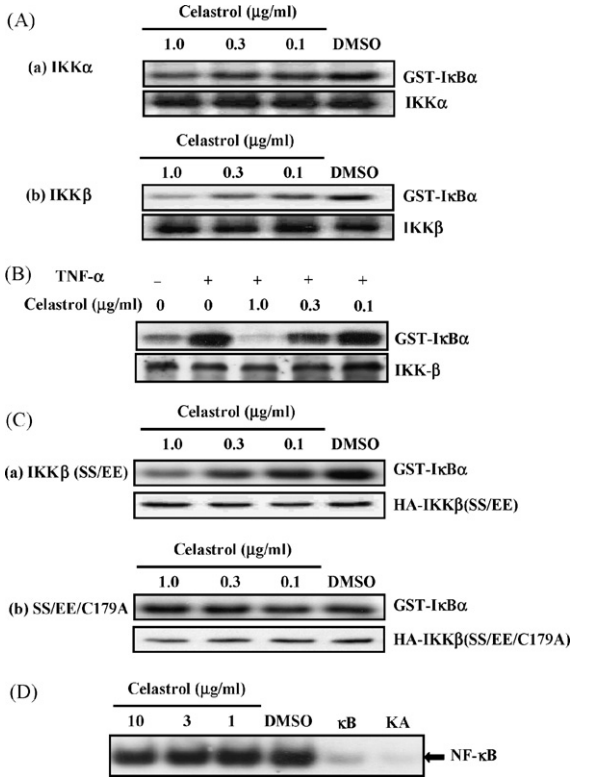

Fig. 4 – Effect of celastrol on the activity for IKK, constitutively active IKKβ (SS/EE), IKKβ (SS/EE/C179A), and the DNA-binding activity of activated NF-κB. (A) IKKα (a) or IKKβ (b) immunocomplex was prepared from 293 cells transfected with IKKα or IKKβ 15 min after TNF-α stimulation. *In vitro* kinase assays were performed with GST-IκBα and [γ-^{32}P]ATP in the presence or absence of various dose of celastrol. To show the equal amount of immunocomplex in each reaction, the bottom represents IKKα or IKKβ detected with Western blot. (B) IKK immunoprecipitates prepared from HeLa cells after TNF-α stimulation for 20 min in the presence of the indicated concentrations of celastrol were conducted *in vitro* kinase assay. To show the equal amount of immunocomplex in each reaction, the bottom represents IKKβ detected with Western blot. (C) HA-IKKβ (SS/EE, a) or HA-IKKβ (SS/EE/C179A, b) immunocomplex was prepared from 293 cells transfected with HA-IKKβ (SS/EE) or HA-IKKβ (SS/EE/C179A). *In vitro* kinase assays were performed with GST-IκBα and [γ-^{32}P]ATP in the presence or absence of various dose of celastrol. To show the equal amount of immunocomplex in each reaction, the bottom represents HA-IKKβ (SS/EE) or HA-IKKβ (SS/EE/C179A) detected with Western blot. (D) The nuclear extracts were prepared from RAW264.7 cells after the stimulation with LPS (1 μg/ml) for 60 min. The indicated amounts of celastrol were directly added to the reaction mixture to determine the effect of the compound on DNA-binding activity of the activated NF-κB. In lane κB; a 100-fold excess of unlabeled NF-κB oligonucleotide was added to the reaction mixture. In lane KA; kamebakaurin (10 μg/ml), a specific inhibitor of DNA-binding activity of p50 subunit of NF-κB [19], was added in the reaction mixture as a control. An arrow indicates the position of specific NF-κB-DNA complex.

of celastrol (Fig. 3C). We observed the same inhibitory effect of celastrol on the LPS- or PMA-induced IκBα phosphorylation as TNF-α (data not shown). These results indicate that celastrol prevents the diverse stimuli-induced IκBα degradation through inhibiting IκBα phosphorylation, thereby interfering with one of the common steps in the signaling cascade leading to the NF-κB activation.

3.4. *Celastrol inhibits IκB kinase*

Since IKK complex acts as a converging point for a variety of upstream activating kinases, we firstly determined if celastrol inhibits IKK activity. IKK immunoprecipitates were prepared from 293 cells transfected with IKKα or IKKβ after TNF-α stimulation, and then *in vitro* kinase assays were conducted in the presence of various concentrations of celastrol (Fig. 4A). Celastrol inhibited IKKα and IKKβ activity in a dose-dependent manner, with IC_{50} values 1.5 and 0.5 μg/ml, respectively. To confirm that celastrol inhibits IKK activity in cells, the endogenous IKK immunocomplexes were prepared from HeLa cells after TNF-α stimulation in the presence of various concentrations of celastrol, and then *in vitro* kinase assays were performed (Fig. 4B). Celastrol significantly inhibited IKK activity in a dose-dependent manner in cells. To exclude the possibility that celastrol prevents IKK activation via inhibition of an upstream kinase, constitutively active IKKβ was tested for sensitivity to celastrol. An epitope-tagged IKKβ derivative (HA-SS/EE IKKβ) was rendered constitutively active by substitution of serine residues 177 and 181 with negatively charged residues (i.e., glutamate) to mimic the activating

phosphorylation at those sites [15,27]. Anti-HA immunoprecipitates prepared from 293 cells transfected with constitutively active IKKβ were found to induce GST-IκBα (1–54) phosphorylation in immunocomplex kinase assays (Fig. 4C). Importantly, this constitutively active IKK activity was inhibited by celastrol in a similar manner as IKKβ activity induced by TNF-α. This result suggested that celastrol inhibits IKK activity by directly targeting rather than inhibiting an activating kinase upstream of IKKβ. This is further supported by the use of an IKKβ derivative mutated at cysteine 179, which lies in the activation loop of the kinase between the two serine residues that are phosphorylated in response to proinflammatory cytokines. Cysteine 179 has recently been shown to be a site for modification by IKKβ inhibitors such as arsenite, prostaglandin, and parthenolide [28–30]. Introduction of the C179A mutation into SS/EE IKKβ rendered a constitutively active IKKβ resistant to 1 μg/ml of celastrol, a concentration that effectively inhibited the kinase activity of SS/EE IKKβ (Fig. 4C). Since many NF-κB inhibitors target cysteine residue in the RelA, p50, and/or IKK through direct modification by a reactive α, β-unsaturated carbonyl group [17,19,29,30], we examined whether the effect of celastrol on the NF-κB activation is abolished by dithiothreitol (DTT). Preincubation of celastrol with DTT reversed the inhibitory effect of celastrol on the LPS-induced NF-κB activation (supplementary data 1). Next we examined whether celastrol directly affects DNA-binding activity of NF-κB (Fig. 4D). Activated NF-κB were prepared from RAW264.7 cells by the stimulation with LPS and incubated with various amount of celastrol *in vitro*, and then DNA-binding activity of the activated NF-κB was measured by EMSA. Kamebakaurin significantly inhibited DNA-binding activity of the activated NF-κB as previously reported [19], however, celastrol did not up to 10 μg/ml. These results suggested that celastrol could directly target cysteine 179 in the activation loop of IKKβ.

3.5. *Celastrol inhibits the expression of anti-inflammatory and anti-apoptotic NF-κB target genes*

Next, we analyzed the effect of celastrol on the expression of NF-κB target genes such as iNOS, TNF-α, and Bfl-1/A1. After RAW264.7 cells were pretreated with various concentration of celastrol for 30 min, cells were stimulated with LPS for 3 h and then the expressions of iNOS and TNF-α were measured by Northern blot analysis. Celastrol significantly suppressed the LPS-induced expression of iNOS and TNF-α in a dose-dependent manner (Fig. 5A). Reduced mRNA expressions of iNOS and TNF-α might be responsible for diminished production of NO and TNF-α in celastrol treated cells. Therefore, the amounts of NO and TNF-α were measured in celastrol treated cells (Fig. 5B and C). RAW264.7 cells were preincubated with various concentrations of celastrol for 30 min and subsequently stimulated with LPS for 20 h, and then the amount of NO and TNF-α secreted into the medium was determined. In consistent with the effects of celastrol on the LPS-induced expressions of iNOS and TNF-α, celastrol inhibited the production of NO and TNF-α dose-dependently. We also analyzed the effect of celastrol on the TNF-α-induced Bfl-1/A1 expression. HT-1080 cells were preincubated with various concentrations of celastrol for 30 min and subsequently stimulated with TNF-α for 3 h, and then the Bfl-1/A1 expression was analyzed by Northern blot. TNF-α significantly

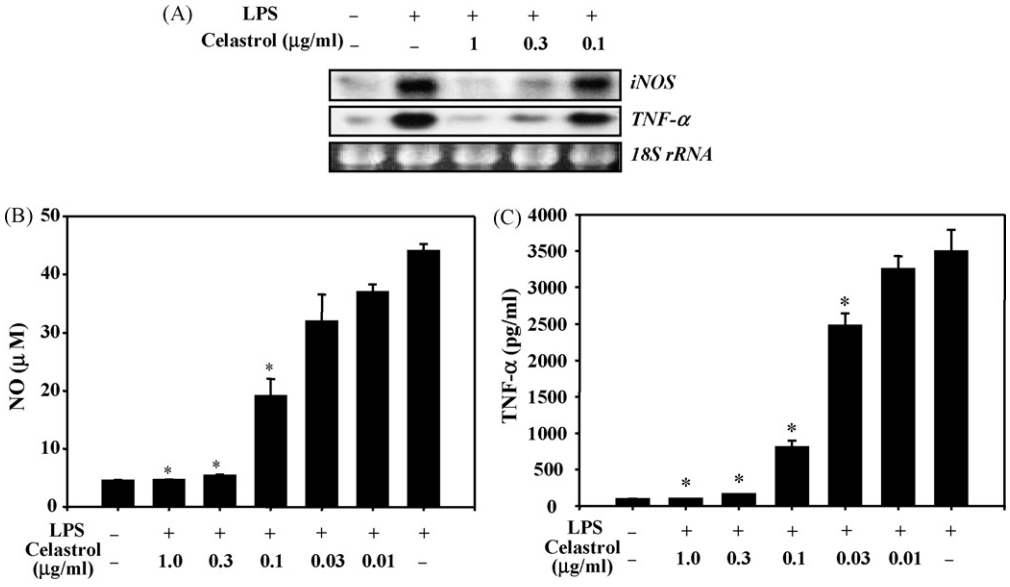

Fig. 5 – Effect of celastrol on the inflammatory NF-κB target genes. (A) RAW264.7 cells were pretreated for 30 min with the indicated concentrations of celastrol and followed by the stimulation with 1 μg/ml of LPS for 3 h. Subsequently, total RNAs were isolated and Northern blot analysis for iNOS and TNF-α expression was performed as described in Section 2. Ethidium bromide staining 18S ribosomal RNA band on the gel was shown to demonstrate equal loading of RNA. (B and C) RAW264.7 cells were pretreated with the indicated concentrations of celastrol for 30 min and treated with LPS (1 μg/ml). After 24 h incubation, the amounts of NO (B) and TNF-α (C) in culture supernatants were measured as described in Section 2. Mean values from two independent experiments performed in triplicated are shown; bar indicates the S.D.s. Statistical significance ($p < 0.01$) judged by paired Student's *t*-test is marked with an asterisk.

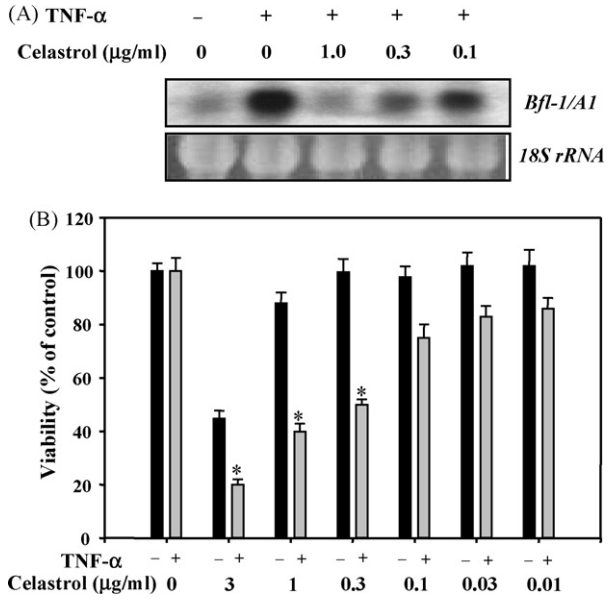

Fig. 6 – Effect of celastrol on the TNF-α-induced cell death. (A) HT-1080 cells were pretreated for 30 min with the indicated concentrations of celastrol and followed by the stimulation with 20 ng/ml of TNF-α for 3 h. Subsequently, total RNAs were isolated and Northern blot analysis for *Bfl-1/A1* expression was performed as described in Section 2. Ethidium bromide staining 18S ribosomal RNA band on the gel was shown to demonstrate equal loading of RNA. (B) HT-1080 cells were pretreated with the indicated concentrations of celastrol for 30 min and treated with TNF-α (20 ng/ml). After 48 h incubation, cell viability was determined by MTT assays. Mean values from two independent experiments performed in triplicated are shown; bar indicates the S.D.s. Statistical significance ($p < 0.01$) judged by paired Student's *t*-test is marked with an asterisk.

induced the Bfl-1/A1 mRNA expression, whose expression is controlled by NF-κB, however, its expression was inhibited by celastrol in a dose-dependent manner (Fig. 6A). Furthermore, co-treatment of celastrol with 20 ng/ml of TNF-α significantly potentiated the cytotoxicity of TNF-α in a dose-dependent manner (Fig. 6B).

3.6. *Anti-inflammatory and anti-tumor activities of celastrol in vivo*

To further extend our observation in conjunction with the recent findings that NF-κB is a key player linking between inflammation and cancer [11,12], we investigated the anti-inflammatory and anti-tumor activities of celastrol in animal models. Firstly, we examined the anti-inflammatory effect of celastrol in an air pouch model of inflammation (Fig. 7). We measured the level of three inflammatory parameters, MPO, TNF-α and PGE_2 in the exudate induced by carrageenan. Administration of celastrol significantly inhibited the level of these parameters *in vivo* in a dose-dependent manner. Administration of celastrol (30 mg/kg) suppressed MPO activity by 90.7 ± 2.1%, TNF-α production by 66.5 ± 3.0%, and PGE_2 production by 93.0 ± 2.0% in the exudate.

To evaluate anti-tumor activity of celastrol, a human breast tumor cell line, MDA-MB-435 cells, were implanted into athymic nude mice. When tumors reached ~100 mm^3 in volume, tumor-bearing mice were treated via i.p. every other day with celastrol to the end of study (Fig. 8). Administration of celastrol significantly suppressed the growth of tumor without the loss of body weight in a dose-dependent manner. Administration of 30 mg/kg of celastrol resulted in 60 ± 5% inhibition of tumor growth compared with the vehicle treated group.

4. Discussion

Plants of the *Celestraceae* family including *Tripterygium wilfordii* and *Celastrus angulatus* are a rich source of quinone methide triterpenoids. Extracts containing quinone methide, celastrol have been used in traditional medicine such as rheumatoid arthritis [21]. It has been reported that celastrol exerts potent anti-inflammatory activities in various experimental models [31] and strong cytotoxicity against various cancer cell-lines [32]. However, how celastrol exerts these pharmacological effects is not clear. Here, we demonstrate for the first time that celastrol, a quinone methide triterpenoid, inhibits NF-κB activation by directly targeting cysteine 179 in the IKK and this feature is likely to contribute to its anti-inflammatory and anti-tumor activities.

Our results show that celastrol strongly blocked the NF-κB activation by various stimuli including TNF-α, PMA, and LPS. Pretreatment of cells with celastrol blocked the IκB phosphorylation and degradation by various stimuli, suggesting that celastrol interferes with one or more common steps during NF-κB activation in different cell types rather than with one single event specific for an individual stimuli. The critical step in NF-κB activation is IκBα phosphorylation at Ser-32 and Ser-36 by IκB kinase complex (IKC) [5]. IKC is found to be activated by a number of different protein kinases including NIK [33] or MEKK-1 [34]. Since celastrol effectively inhibited not only IκBα phosphorylation in response to various stimuli but also NF-κB activation by MEKK-1 and IKKβ, but RelA, it was important to determine if celastrol directly inhibits IKK or the activity of upstream kinase activity. Indeed, celastrol effectively inhibits the activity for both IKKs and a constitutive active IKKβ (SS/EE mutant). Number of natural NF-κB inhibitors such as cyclopentenone prostaglandin, sequiterpene parthenolide, and diterpene kamebakaurin target critical cysteine residue in the RelA, p50, and/or IKK through direct modification by a reactive α, β-unsaturated carbonyl group [29,30,19]. Celastrol also contains a reactive quinone methide moiety extended by one additional double bond and inhibited even a constitutive active IKKβ (SS/EE mutant) activity. These observations led us to hypothesize that celastrol may directly modify a critical cysteine residue in the IKKα and IKKβ. Both of the IKKs contain a cysteine at 179 in their activation loop. To examine whether this cysteine is a critical for sensitivity to celastrol, constitutively active IKKβ (SS/EE mutant) and its mutant IKKβ (SS/EE/C179/A) were examined their sensitivity toward celastrol. Indeed, a single amino acid substitution (C179A) in the kinase

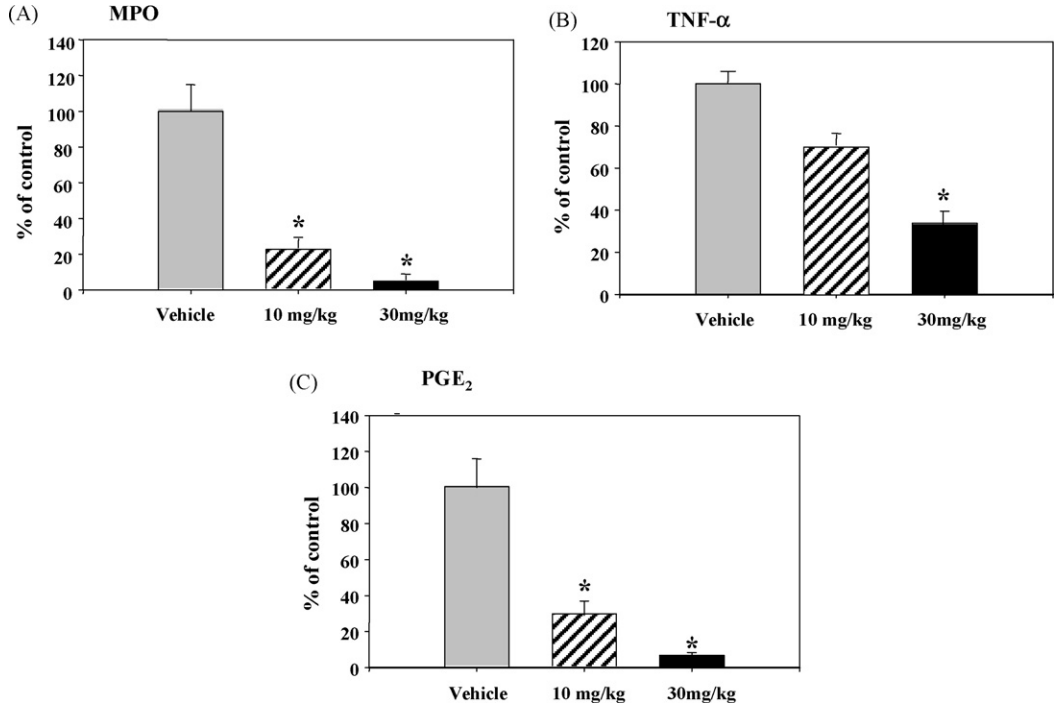

Fig. 7 – Anti-inflammatory activity of celastrol in mice. Effect of celastrol on the air pouch model of inflammation. Myeloperoxidase activity (A), the amount of TNF-α (B), and PGE_2 (C) in the exudates were measured as described in Section 2. Mean values from six mice per group are shown; bar indicates the S.D.s. Statistical significance ($p < 0.01$) judged by ANOVA test is marked with an asterisk.

activation loop of the constitutively active IKKβ renders it insensitive to celastrol. Given the lack of celastrol sensitivity in the C179A mutant along with the inactivation of celastrol by DTT (supplementary data 1), this cysteine residue is a likely candidate for the site of covalent modification by celastrol as shown by other compounds [28–30]. However, different cysteine targets such as cysteine 62 of p50 and cysteine 38 of RelA, which are important for DNA binding of p50 and RelA,

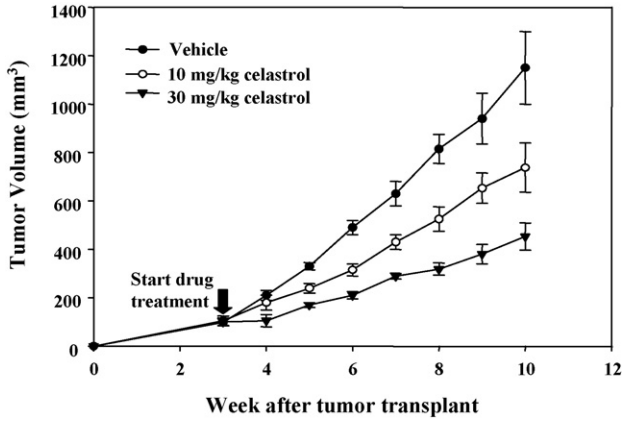

Fig. 8 – Effects of celastrol in nude mice bearing MDA-MB-435 xenografts. Intraperitoneal administration of celastrol or vehicle was initiated when tumors reached an average of ~100 mm^3. Tumor volume was measured on the indicated days. Mean tumor volume for each group from eight mice is shown; bar indicates S.E.

have been demonstrated for 15d-PGJ_2 and parthenolide, respectively [29,30]. On the other hand, celastrol is likely to selectively modify only cysteine 179 of IKK because it did neither inhibit the NF-κB activation induced by RelA over-expression nor directly suppressed the DNA-binding activity of activated NF-κB (Fig. 4D). This selectivity could be attributed to structural difference of the quinone methide unsaturated system in celastrol from those in 15d-PGJ_2 and parthenolide. Recently, it has been reported that celastrol induces heat shock protein 70 (HSP70) expression, which disrupts NF-κB activation by indirectly regulating the assembly of the IKC via interacting with IKKγ, however, this induction is independent on its quinone methide moiety [35,36]. On the other hand, reduction of the quinone methide in celastrol with $NaBH_4$ to dihydrocelastrol significantly reduced its inhibitory effect on NF-κB activation (supplementary data 2), suggesting that celastrol could inhibit NF-κB activation by directly targeting IKK rather than indirectly inducing HSP70. Various pharmacological effects of celastrol have been reported. Celastrol has lipid anti-peroxidation [37] and inhibitory activity of IL-1β production induced by LPS [38]. It suppresses adjuvant arthritis and significantly improved memory, learning and psychomotor activity tests in the rat without toxicity [31]. Pristimerin, an analogue of celastrol, was reported to have inhibitory activity of induced iNOS expression and DNA-binding activity of NF-κB by LPS [39]. Recently, it has also been reported that celastrol suppresses the production of pro-inflammatory cytokines by inhibiting the nuclear translocation of NF-κB and the phosphorylation of p38 kinase in Crohn's disease biopsies [40]. Our results, however, clearly demonstrates molecular target of celastrol in the NF-κB

signaling pathway, its effect on proinflammatory protein iNOS and TNF-α expression, and a dose-dependent *in vivo* efficacy in the air pouch model.

The NF-κB is definitely a key player in inflammation. It now seems that it might also activate signaling pathways in both cancer cells and tumor-associated inflammatory cells, which promote tumor malignancy. The role of NF-κB in inflammation and cancer has been extensively studied. Furthermore, recent two studies show that NF-κB pathway plays a crucial role in a link between inflammation and cancer [11,12]. Moreover, several NSAIDs used in chemoprevention of colorectal cancer can act as IKKβ inhibitors [15,16]. Anti-apoptotic activity of NF-κB involves the inhibition of TNF-α-induced apoptosis through induction of a variety of anti-apoptotic proteins including cIAPs, Bcl-XL, and Gadd45β [10]. It is therefore possible that anti-tumor activity of celastrol might be, at least in part, connected to its NF-κB inhibitory effect, of which suppresses Bfl-1/A1 expression, thereby potentiates TNF-induced cytotoxicity. In this regard, our findings that celastrol suppressed IKKβ activity and exerted anti-inflammatory and anti-tumor activities extend our understanding on the molecular mechanisms underlying the anti-inflammatory and anti-tumor activities of celastrol and celastrol-containing extracts that are used in traditional oriental medicine. Furthermore, celastrol could be an interesting lead compound for the modulation of inflammatory diseases as well as certain cancers in which inhibition of NF-κB-activity may be desirable.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2006.08.014.

REFERENCES

[1] Ghosh S, May MJ, Kopp EB. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 1998;16:225–60.
[2] Baldwin Jr AS. The transcription factor NF-κB and human disease. J Clin Invest 2001;107:3–6.
[3] Huxford T, Huang DB, Malek S, Ghosh G. The crystal structure of the IκB/NF-κB complex reveals mechanisms of NF-κB inactivation. Cell 1998;95:759–70.
[4] Jacobs MD, Harrison SC. Structure of an IκB/NF-κB complex. Cell 1998;95:749–58.
[5] Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li JW, et al. IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. Science 1997;278:860–5.
[6] Woronicz JD, Gao X, Cao Z, Rothe M, Goeddel DV. IκB kinase-β: NF-κB activation and complex formation with IκB kinase-α and NIK. Science 1997;278:866–9.
[7] Chen Z, Hagler J, Palombella V, Melandri F, Scherer D, Ballard D, et al. Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. Genes Dev 1995;9:1586–97.
[8] Scherer DC, Brockman JA, Chen A, Maniatis T, Ballard DW. Signal-induced degradation of IκBα requires site-specific ubiquitination. Proc Natl Acad Sci USA 1995;92:11259–63.
[9] Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994;12:141–79.
[10] Karin M, Greten FR. NF-κB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 2005;5:749–59.
[11] Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, et al. IKKβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 2004;118:285–96.
[12] Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, et al. NF-κB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–6.
[13] Karin M, Yamamoto Y, Wang QM. The IKK-NF-κB system: a treasure trove for drug development. Nat Rev Drug Discov 2004;3:17–26.
[14] De Bosscher K, Schmitz ML, Plaisance S, Vanden Berghe W, Fiers W, Haegeman G. Glucocorticoid-mediated repression of nuclear factor-κB-dependent transcription involves direct interference with transactivation. Proc Natl Acad Sci USA 1997;94:13504–9.
[15] Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. Nature 1998;396:77–80.
[16] Yamamoto Y, Yin MJ, Lin KM, Gaynor RB. Sulindac inhibits activation of the NF-κB pathway. J Biol Chem 1999;274:27307–14.
[17] Hehner SP, Heinrich M, Bork PM, Vogt M, Ratter F, Lehmann V, et al. Sesquiterpene lactones specifically inhibit activation of NF-κB by preventing the degradation of IκB-α and IκB-β. J Biol Chem 1998;73:1288–97.
[18] Lyss G, Knorre A, Schmidt TJ, Pahl HL, Merfort I. The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-κB by directly targeting p65. J Biol Chem 1998;273:33508–16.
[19] Lee JH, Koo TH, Hwang BY, Lee JJ. Kaurane diterpene, kamebakaurin, inhibits NF-κB by directly targeting the DNA-binding activity of p50 and blocks the expression of antiapoptotic NF-κB target genes. J Biol Chem 2002;277:18411–20.
[20] Jin HZ, Hwang BY, Kim HS, Lee JH, Kim YH, Lee JJ. Antiinflammatory constituents of *Celastrus orbiculatus* inhibits the NF-κB activation and NO production. J Nat Prod 2002;65:89–91.
[21] Gunatilaka AAL. Triterpenoid quinonemethides and related compounds (celastroloids). In: Herz W, Kirby GW, Moore RE, Steglich W, Tamm CH, editors. Progress in the chemistry of organic natural products, vol. 67. Vienna: Springer; 1996. p. 1–176.
[22] Ngassapa O, Soejarto DD, Pezzuto JM, Farnsworth NR. Quinone-methide triterpenes and salaspermic acid from *Kokoona ochracea*. J Nat Prod 1994;57:1–8.
[23] Schmidt HHHW, Kelm M. Determination of nitrite and nitrate by the Griess reaction. In: Methods in nitric oxide research. London: John Wiley & Sons Ltd.; 1996. p. 491–7.
[24] Romano M, Faggioni R, Sironi M, Sacco S, Echtenacher B, Di Santo E, et al. Carrageenan-induced acute inflammation in the mouse air pouch synovial model. Role of tumour necrosis factor. Mediators Inflamm 1997;6:32–8.

[25] Lee JH, Choi JK, Noh MS, Hwang BY, Hong YS, Lee JJ. Anti-inflammatory effect of kamebakaurin in *in vivo* animal models. Planta Med 2004;70:526–30.
[26] Lee JH, Welch DR. Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1. Cancer Res 1997;57:2384–7.
[27] Delhase M, Hayakawa M, Chen Y, Karin M. Positive and negative regulation of IκB kinase activity through IKKβ subunit phosphorylation. Science 1999;284:309–13.
[28] Kapahi P, Takahashi T, Natoli G, Adams SR, Chen Y, Tsien RY, et al. Inhibition of NF-κB activation by arsenite through reaction with a critical cysteine in the activation loop of IκB kinase. J Biol Chem 2000;275:36062–6.
[29] Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, et al. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase. Nature 2000;403:103–8.
[30] Kwok BHB, Koh B, Ndubuisi MI, Elofsson M, Crews CM. The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits IκB kinase. Chem Biol 2001;8:759–66.
[31] Allison AC, Cacabelos R, Lombardi VR, Alvarez XA, Vigo C. Celastrol, a potent antioxidant and anti-inflammatory drug, as a possible treatment for Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry 2001;25:1341–57.
[32] Chang FR, Hayashi K, Chen IH, Liaw CC, Bastow KF, Nakanishi Y, et al. Antitumor agents. 228. Five new agarofurans, reissantins A–E, and cytotoxic principles from *Reissantia buchananii*. J Nat Prod 2003;66:1416–20.
[33] Malinin NL, Boldin MP, Kovalenko AV, Wallach D. MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. Nature 1997;385:540–4.
[34] Lee FS, Peters RT, Dang LC, Maniatis T. MEKK1 activates both IκB kinase α and IκB kinase β. Proc Natl Acad Sci USA 1998;95:9319–24.
[35] Westerheide SD, Bosman JD, Mbadugha BN, Kawahara TL, Matsumoto G, Kim S, et al. Celastrols as inducers of the heat shock response and cytoprotection. J Biol Chem 2004;279:56053–60.
[36] Ran R, Lu A, Zhang L, Tang Y, Zhu H, Xu H, et al. Hsp70 promotes TNF-mediated apoptosis by binding IKKγ and impairing NF-κB survival signaling. Genes Dev 2004;18:1466–81.
[37] Sassa H, Takaishi Y, Terada H. The triterpene celastrol as a very potent inhibitor of lipid peroxidation in mitochondria. Biochem Biophys Res Commun 1990;172:890–7.
[38] He W, Huang FC, Gavai A, Chan WK, Amato G, Yu KT, et al. Novel cytokine release inhibitors. Part III. Truncated analogs of tripterine. Bioorg Med Chem Lett 1998;8:3659–64.
[39] Dirsch VM, Kiemer AK, Wagner H, Vollmar AM. The triterpenoid quinonemethide pristimerin inhibits induction of inducible nitric oxide synthase in murine macrophages. Eur J Pharmacol 1997;336:211–7.
[40] Pinna GF, Fiorucci M, Reimund JM, Taquet N, Arondel Y, Muller CD. Celastrol inhibits pro-inflammatory cytokine secretion in Crohn's disease biopsies. Biochem Biophys Res Commun 2004;322:778–86.