Immunopharmacology and Inflammation

Celastrol binds to ERK and inhibits FcεRI signaling to exert an anti-allergic effect

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Abstract

The role of celastrol, a triterpene extracted from the Chinese “Thunder of God Vine,” in allergic inflammation was investigated. Celastrol decreased the secretion of β-hexosaminidase, decreased the release of histamine, decreased the expression of Th2 cytokines and decreased calcium influx and cell adhesion in antigen-stimulated RBL2H3 cells. Exposure to celastrol decreased the phosphorylation of extracellular regulated kinase (ERK) and the ERK kinase activity was decreased in RBL2H3 cells. A molecular dynamics simulation showed binding of celastrol to a large pocket in ERK2, which serves as the ATP-binding site. Exposure to celastrol inhibited the interaction between immunoglobulin Fc epsilon receptor I (FcεRI) and ERK and inhibited interaction between FcεRI and protein kinase Cδ (PKCδ). Antigen stimulation induced an interaction between Rac1 and ERK as well as an interaction between Rac1 and PKCδ. Inhibition of ERK decreased Rac1 activity and inhibition of Rac1 decreased ERK activity in antigen-stimulated RBL2H3 cells. Celastrol regulated the expression of epithelial–mesenchymal transition (EMT)-related proteins via inhibition of PKC/Rac1 signaling. Celastrol exerted a negative effect on in vivo atopic dermatitis induced by 2, 4-dinitrofluorobenzene (DNFB), which requires ERK. Celastrol also showed an inhibitory effect on skin inflammation induced by phorbol myristate acetate (PMA) in Balb/c mice. In summary, celastrol binds to ERK and inhibits FcεRI signaling to exert an anti-inflammatory effect.

Keywords:
Allergic inflammation
Celastrol
Extracellular regulated kinase
Heat shock protein 90
FcεRI

1. Introduction

Celastrol, a quinine methide triterpene derived from the medicinal plant Trypterigium wilfordii has been used to treat chronic inflammatory and autoimmune diseases.

Celastrol has shown promise as an anti-inflammatory compound in rat models and for the treatment of human of collagen-induce arthritis and rheumatoid arthritis (Tao et al., 2002), systemic lupus erythematosus (Li et al., 2005), and Crohn’s disease (Pinna et al., 2004). Celastrol has been shown to induce the expression of heat shock proteins (Chow and Brown, 2007). Celastrol has been shown to inhibit cytotoxicity through induction of the heat shock response (Zhang and Sarge, 2007). Celastrol has been shown to act as an anti-inflammatory molecule by exerting a negative effect on cytokine-induced adhesion molecule expression and adhesiveness in human endothelium (Zhang et al., 2006). Celastrol has been shown to exert an anti-inflammatory effect by the inhibition of NF-κB activity and NO production (Jin et al., 2002).

Based on the potential role of celastrol in inflammation, we examined effect of celastrol on allergic inflammation. Celastrol was found to inhibit immunoglobulin Fc epsilon receptor I (FcεRI) signaling that involved protein kinase C (PKCδ), PKCδ, Rac1, extracellular regulated kinase (ERK), Akt, and heat shock protein 90 (hsp90). PKCδ, Rac1, and ERK constitute FcεRI signaling and Akt and Lyn constitute FcεRI signaling. Celastrol was found to bind to ERK2 and inhibits FcεRI signaling. Celastrol also exerted a negative effect on FcεRI signaling by disrupting the interaction between hsp90 and proteins such as FcεRIβ, Akt and PKCδ. Celastrol prevented the antigen from regulating the expression of epithelial–mesenchymal transition (EMT)-related proteins via inhibition of PKC/Rac1 signaling. Celastrol exerted an in vivo anti-allergic effect in an atopic dermatitis model of Nc/Nga mice and Balb/c mice.

2. Materials and methods

2.1. Materials

Primers used in this study were commercially synthesized by Bionex (Seoul, Korea). Dinitrofenyl (DNP)-Human serum albumin
(HSA) and DNP-specific IgE were purchased from Sigma Chemical Company. Chemicals used in this study were purchased from Sigma Chemical Company. Anti-ERK, anti-phospho ERK, anti-E-cadherin, anti-snail, anti-vimentin, and anti-N-cadherin antibodies were purchased from R&D Company (Minneapolis, MN). All other antibodies used in this study were purchased from Cell signaling Company. Anti mouse and anti-rabbit IgG–horse radish peroxidase-conjugate antibody was purchased from Pierce Company (Rockford, IL). Cytokine ELISA kits were purchased from Koma Biotech (Korea).

2.2. Cell culture

RBL2H3 cells were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown in Dulbecco’s modified Eagle’s medium containing heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, San Diego, CA). Cultures were maintained in 5% CO2 at 37 °C.

2.3. Mice

Five week-old female Balb/c and Nc/Nga mice were purchased from SLC Japan (Shizuoka, Japan) and maintained in SPF condition. All animal experiments were approved by the Institutional Review Board for animal studies of Kangwon National University. At day 25, blood samples were drawn for the measurement of serum immunoglobulin levels.

2.4. β-hexosaminidase secretion assay

Degranulation was determined by measuring the release of β-hexosaminidase (Matsubara et al., 2004). To determine the effect of ERK or metalloprotease-2 (MMP-2) on β-hexosaminidase secretion, IgE-sensitized RBL2H3 cells were pretreated with PDB08059 (20 μM) or GM6001 (1 μM) for 15 min. Cells were then stimulated with DNP-HSA (100 ng/ml) for 1 h. Secretion of β-hexosaminidase was then measured.

2.5. 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay

Cellular toxicity of celastrol was examined using an XTT assay (Cell proliferation assay kit II, Roche Molecular. Biochemicals, Mannheim, Germany). In brief, RBL2H3 cells were grown in a 5% CO2 atmosphere in 96-well plates in 100 μl culture medium and incubated for various time periods at 37 °C in the presence or absence of various concentrations of celastrol for 15 and 30 min. After celastrol treatment, 50 μl of XTT labeling mixture was added to each well and further incubated at 37 °C for 8 to 24 h. Absorbances were measured at 450 nm with the use of a microplate reader.

2.6. Histamine release assay

Histamine release assays in guinea pig mast cells were also carried out. In brief, guinea pig mast cells were isolated, and purified by enzyme digestion and rough and discontinuous Percoll density gradient method. Mast cells (4 × 10^7 cells) were passively sensitized by anti-ovalbumin antibody, and challenged by 1.0 μg/ml ovalbumin. Celastrol (10 μM) was added 5 min before antigen challenge. Histamine in supernatant was determined by fluorometric analyzer.

Histamine release in antigen-stimulated RBL2H3 cells was analyzed by protocol supplied by manufacturer (Immunotech, France).

2.7. The effect of celastrol on calcium influx

RBL2H3 cells were sensitized for 16 h with DNP-specific IgE (100 ng/ml) on 2% (w/v) BSA-coated 6 well plates. The growth medium was then replaced with assay buffer, and the cells were exposed to 4 μM fluo-3–Acetoxyxethyl (AM) esters in the presence of 1% (v/v) fetal bovine serum and 0.04% Pluronic F-127 in the dark for 45 min. The fluo-3–loaded cells were washed three times with assay buffer, treated with celastrol (10 μM) for 5 min, and then immediately stimulated with DNP-HSA (100 ng/ml). The change in fluorescence of fluo-3 was monitored at an excitation wavelength of 488 nm and emission wavelength of 540 nm.

2.8. Cell adhesion assays

The IgE-sensitized RBL2H3 cells were pretreated without or with celastrol (10 μM) for 15 min. Cells were then stimulated with DNP-HSA (100 ng/ml) for 1 h. Cells (5 × 10^3) suspended in DMEM, containing 0.5% BSA, were dispensed into 24-well plates that had been coated with fibronectin (10 μg/cm^2 each), incubated in 5% CO2 at 37 °C for 1 h or were coated with 3% BSA and washed three times with PBS. Attached cells were stained with hematoxylin and eosin reagent and counted under light microscopy.

2.9. ERK kinase activity assay

The effect of celastrol on ERK kinase activity was determined using a p42/44 MAP kinase assay kit (Cell Signaling Technology Inc., Danvers, MA, USA). In brief, IgE-sensitized RBL2H3 cells were pretreated with or without celastrol (10 μM) for 15 min, cells were then stimulated with DNP-HSA (100 ng/ml) for 1 h. Cells were washed once with ice-cold PBS, then lysis buffer added to each plate, and the cells were scraped and transferred to fresh tubes. Cells were sonicated on ice twice and centrifuged for 10 min at 4 °C. Protein concentration of the supernatant was determined by Bradford method (Bio-Rad, Munich, Germany). Cell lysate protein (200 μg) and immobilized phospho-p42/44 MAP kinase (Thr-202/Tyr-204) antibody beads (20 μl) were mixed by gently rocking for 3 h at 4 °C. These tubes were centrifuged and washed twice. The pellets were suspended in 20 μl of kinase buffer supplemented with 200 μM ATP and 2 μg Elk-fusion protein and incubated for 45 min at 35 °C. The reaction was terminated with 10 μl of 3× SDS sample buffer. The samples were boiled and loaded for 10% SDS-PAGE. ERKs kinase activity was analyzed by Western blotting using a phospho-Elk1 antibody.

2.10. PKCo kinase assay

Determination of PKCo activity was performed with HTScan® PKCo kinase assay kit (Cell Signaling Technology Inc., Danvers, MA, USA) following the manufacturer’s instruction. The cells were lysed in kinase lysis buffer [25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 10 mM NaF, 10 mM p-nitrophenyl phosphate, 1 mM Na3VO4, 2 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml peptatin, and 1% Triton]. The supernatant was obtained by centrifugation at 13,000 g for 10 min at 4 °C. Protein concentration of the supernatant was determined by Bradford method (Bio-Rad, Munich, Germany). To determine PKCo activity, 10–20 μg cellular proteins were incubated with biotinylated peptide substrate in kinase buffer [25 mM Tris–HCl (pH 7.5), 10 mM MgCl2, 5 mM β-glycerophosphate, 2 mM DTT, 200 μM ATP]. After incubation for 30 min, the reaction was stopped by adding stop buffer (50 mM EDTA, pH 8.0). Reaction mixture (25 μl) was added to a 96 well streptavidin-coated plate (Pierce, Rockford, IL) and incubated for 1 h, followed by washing three times with wash buffer (PBS, 0.05% Tween-20). Phosphospecific substrate antibody was added to each well, samples were incubated for 1 h. After washing three times, the samples were incubated with HRP-labeled secondary antibody for 30 min and washed five times. Tetramethylbenzidine (TMB) substrate was added and incubated for 15 min. The reaction was stopped with...
stop solution and the absorbance at 450 nm was determined with the use of a microplate reader.

2.11. Preparation of siRNA duplexes

The siRNA duplexes were constructed with the following target sequences. Snail, sense (5′-AACCTGCAAAATTGTGCGGGCCTCT-3′); antisense (5′-AACCTGCAAAATTGTGCGGGCCTCT-3′); control, sense (5′-AACCTGCAAAATTGTGCGGGCCTCT-3′); antisense (5′-AACCTGCAAAATTGTGCGGGCCTCT-3′). The construction of siRNA was carried out according to the instruction manual provided by the manufacturer (Ambion, Austin, TX).

2.12. Gelatin zymography

Conditioned medium from RBL2H3 cells cultured in serum-free medium was mixed 3:1 with substrate gel sample buffer [40% (v/v) glycerol, 0.25 M Tris–HCl, pH 6.8, and 0.1% (w/v) bromophenol blue] and loaded onto a 7.5% SDS-PAGE containing type I gelatin (2 mg/ml). After electrophoresis, the gel was soaked in Triton X-100 with shaking for 30 min with one change of solution. The gel was rinsed and incubated for 24 h at 37 °C in substrate buffer [50 mM Tris–HCl, pH 7.5, 5 mM CaCl2, and 0.02% NaN3]. After incubation, the gel was stained with 0.1% Coomassie brilliant blue G-250 and destained in 50% methanol 10% acetic acid and 40% water. To determine the effect of snail on the secretion and induction of MMP-2, cells were transiently transfected with control siRNA (10 nM) or snail siRNA (10 nM). Gelatin zymography was performed as described.

2.13. Rac1 activity assays

Rac1 activity assays were performed according to the well established procedures (Kim et al., 2008). Approximately 10 µg of total lysates was subjected to western blot analysis. To determine the effect of PKC on Rac1 activity, IgE-sensitized RBL2H3 cells were pretreated with rottlerin (10 nM) for 1 h. Gelatin was added to the samples for 4 h, the reaction was stopped by adding 2 M sulfuric acid to the wells. The plates were read at 450 nm using a microtiter plate reader. OD readings were converted to pg/ml using a standard curve and the appropriate dilution factor. IL-5 ELISA assay was performed identically to the IL-13 ELISA.

2.14. Western blot analysis

For polyacrylamide gel electrophoresis (PAGE) and Western blot, cell lysates were prepared using lysis buffer [62.5 mM Tris–HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM DTT, 0.01% (w/v) bromophenol blue, 10 mM NaF, 1% (v/v) protease inhibitor cocktail, 1 mM sodium orthovanadate]. The samples were boiled for 5 min, and equal amounts of protein (20 µg/well) were analyzed on a 10% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and subjected to immunoblotting. The primary antibodies were used in various dilutions suggested by manufacturers. After extensive washing, blots were further incubated with an anti-mouse or anti-rabbit IgG-horseradish peroxidase-conjugate antibody at a 1:3000 dilution for 1 h at room temperature and were developed using an enhanced chemiluminescence kit (Amer sham). For immunoprecipitation, cell lysates were immunoprecipitated with respective antibody (each at 5 µg) on ice for 1 h. Protein G-sepharose was then added and the reaction was performed at 4 °C for 2 h on a rotary shaker. Immune complexes were washed three times with lysis buffer, and 2× sample buffer was added to the beads. Boiled samples were then loaded on gels. Western immunoblot analyses were followed according to standard procedures. For densitometric analyses, NIH J program was employed.

2.15. Cytokine ELISA

Assays were performed according to manufacturer’s instruction (Koma Biotech, Korea). Briefly, anti-Mouse IL-13 Ab was coated overnight at 1 µg/ml on flat-bottomed microtiter plates. The plates were washed twice with PBS, 0.02% (v/v) Tween 20 (Sigma) and incubated for 1 h with PBS containing 1% BSA. After washing, serum samples (1:10 dilution) from Nc/Nga mice, Balb/c mice, and culture media from antigen-stimulated RBL2H3 cells were added to the plates and incubated for 2 h. After washing, biotinylated purified anti-Mouse IL-13 Ab (0.25 µg/ml) was added and incubated for 2 h. The plates were washed three times and incubated with avidin–HRP conjugate (1:2000 dilutions) for 30 min. The reaction was stopped by adding a 2 M sulfuric acid to the wells. The plates were read at 450 nm using a microtiter plate reader. OD readings were converted to pg/ml using a standard curve and the appropriate dilution factor. IL-5 ELISA assay was performed identically to the IL-13 ELISA.

2.16. DNFB-induced dermatitis

Allergic dermatitis was induced by repeated topical application with DNFB. Briefly, 100 µl of 0.15% DNFB in acetone/olive oil (3:1) was applied to shaved back side mice twice at 7 day intervals. DNFB was also applied to each side of right ears of mice twice at 7 day intervals. Ear thickness was measured once a week using a digital thickness gauge. Increase in ear thickness was determined by subtracting ear thickness before the first painting from that of each time point. To determine the effect of celastrol, 50 µg of celastrol was added along with DNFB. 10 µM of 2′-Amino-3′-methoxyflavone (PD98059) was added along with DNFB to determine the effect of ERK on DNFB-induced atopic dermatitis.

2.17. Induction of skin inflammation in BALB/c mice

12-O-tetradecanoylphorbol-13-acetate (PMA) was used as an inducer of skin inflammation. The thickness of both ears of untreated mice was measured using dial thickness gauge (Mitutoyo Corporation, Kanakawa, Japan) as a reference. 20 µl of celastrol (5%) or acetone olive oil (AOO) was pretreated for 30 min by topical application, and 20 µl of PMA (0.015%) was painted to dorsal surface of both ears to induce skin inflammation. After 4 h, the topical application of inducer, ear thickness was measured again and changes in ear thickness were calculated.

2.18. IL-5 and IL-13 secretion of splenocytes

Total splenocytes were plated at 1×10^6 cells/ml and celastrol (5%) was pretreated for 1 h before being incubated with concanavalin A (1 µg/ml) for 48 h. Culture supernatants were collected and the amount of secreted IL-5 and IL-13 was measured by enzyme-linked immunosorbent assay using a protocol supplied by ebioScience Inc (Court West San Diego, USA) and KOMA BIOTECH Inc (Seoul, KOREA), respectively.

2.19. Histological analyses

Skin samples were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 4 µm, and then stained with hematoxylin and eosin. Ear samples were fixed and stained as was done with skin samples.

2.20. Docking simulations

The structure of ERK2 was extracted from 2oji.pdb. Coordinates of celastrol were built by a graphical method of PRODRG (Schuettelkopf and van Aalten, 2004). Docking simulations were performed using Autodock 3 with a Lamarckian genetic algorithm (Morris et al., 1998). The docked structure of ERK2-celastrol complex was refined further by energy minimization using a Gromacs 3.3.0 molecular dynamics simulation software (Lindahl et al., 2001). Briefly, the topology of
Celastrol for the gmx force field was obtained from PRODRG and incorporated in the protein topology. Then the whole system was immersed in a periodic box of flexible SPC water molecules. Electrostatic energy was calculated using the particle mesh Ewald method. Cutoff distances for the calculation of the Coulomb and van der Waals interaction were 1.0 and 1.4 nm, respectively. After energy minimization using a steepest decent method, the system was subject to equilibration for 100 ps under the conditions of position restraints for heavy atoms and LINCS constraints for all bonds. The system was coupled to the external bath by the Berendsen pressure and temperature coupling. The final MD calculations were performed under the same conditions except that the position restraints were removed.

3. Results

3.1. Celastrol exerts an inhibitory effect on calcium influx, β-hexosaminidase secretion, histamine release and cell adhesion in antigen-stimulated RBL2H3 cells

As treatment with celastrol was shown to be effective for some inflammatory diseases, we examined if celastrol exerted a negative effect on allergic inflammation in vitro. Treatment with celastrol caused decreased secretion of β-hexosaminidase from antigen-stimulated RBL2H3 cells in a dose-dependent manner (Fig. 1A). Ketotifen fumarate (KF), an anti-inflammatory drug, also decreased the secretion of β-hexosaminidase (Fig. 1A). The effect of celastrol on mediator release from antigen-stimulated mast cells was determined. Exposure to celastrol decreased histamine levels from ovalbumin-stimulated guinea pig lung mast cells (Fig. 1B). Celastrol decreased the adhesion of antigen-stimulated RBL2H3 cells to extracellular matrix protein (Fig. 1C). However, BSA did not enhance adhesion of IgE-sensitized RBL2H3 cells to extracellular matrix protein (Fig. 1C). Celastrol, at various concentrations (0.01, 0.1, 1, 5, 10 µM), did not show any cytotoxic effect in RBL2H3 cells (data not shown).

3.2. Celastrol decreases the secretion of β-hexosaminidase, histamine release and Th2 cytokine expression by the inhibition of ERK activation in antigen-stimulated RBL2H3 cells

Mitogen activated protein kinase (MAPK) signaling has been shown to be an essential mediator of allergic inflammation. Therefore, the effect of celastrol on MAPK signaling was investigated. Treatment with celastrol decreased the phosphorylation of ERK in antigen-stimulated RBL2H3 cells in a dose-dependent manner (Fig. 2A). We examined whether celastrol inhibited ERK kinase activity by measuring the phosphorylation of Elk-1, a downstream target of ERK. As treatment with celastrol was shown to be effective for some inflammatory diseases, we examined if celastrol exerted a negative effect on allergic inflammation in vitro. Treatment with celastrol caused decreased secretion of β-hexosaminidase from antigen-stimulated RBL2H3 cells (Fig. 2A). Inhibition of ERK by 2′-Amino-3′ methoxyflavone (PD98059) (10 µM) decreased the secretion of β-hexosaminidase from antigen-stimulated RBL2H3 cells (Fig. 2B). Ketotifen fumarate, an anti-allergic drug, also decreased the secretion of β-hexosaminidase from antigen-stimulated RBL2H3 cells (Fig. 2B). Ketotifen fumarate, an anti-allergic drug, also decreased the secretion of β-hexosaminidase from antigen-stimulated RBL2H3 cells (Fig. 2B). Ketotifen fumarate, an anti-allergic drug, also decreased the secretion of β-hexosaminidase from antigen-stimulated RBL2H3 cells (Fig. 2B). Ketotifen fumarate, an anti-allergic drug, also decreased the secretion of β-hexosaminidase from antigen-stimulated RBL2H3 cells (Fig. 2B).

3.3. Molecular dynamics simulation shows binding of celastrol to a large pocket in ERK2

Since celastrol inhibited the phosphorylation of ERK and inhibited ERK kinase activity in antigen-stimulated RBL2H3 cells, it was of interest to examine if celastrol was bound to ERK. For docking simulations, the carboxy group of celastrol was assumed to be ionized based on its low pK. Typically, more than 90% of the docked conformations were clustered into a single group (rmsd 0.5 nm) with the lowest docking energy (−11.37 kcal/mol). This finding suggests that the binding is highly specific. Moreover, the docked

Fig. 1. Celastrol exerts an inhibitory effect on calcium influx, β-hexosaminidase secretion, histamine release and cell adhesion in antigen-stimulated RBL2H3 cells. (A) The enzyme activities of β-hexosaminidase in the supernatant and solubilized cells were measured with the use of p-nitrophenyl N-acetyl-beta-D-glucosamine. Statistical analyses were carried out by the use of student t test. **P<0.005 compared with RBL2H3 cells without antigen stimulation; ***P<0.0005 compared with RBL 2H3 cells without antigen stimulation; +P<0.05 compared with antigen stimulated cell untreated with celastrol; **P<0.005 compared with antigen stimulated cells untreated with celastrol. M denotes mock. D denotes DMSO. KF denotes ketotifen fumarate. (B) Guinea pig mast cells were isolated and were purified by enzyme digestion and by use of the rough and discontinuous percoll density gradient method. Mast cells (4×10⁵ cells) were passively sensitized by an anti-ovalbumin antibody and were challenged by 1.0 µg/ml ovalbumin. Celastrol, at the indicated concentration, was added 5 min before antigen challenge. Histamine in supernatant was determined by fluorometric analyzer. **P<0.005 compared with ovalbumin-stimulated guinea pig mast cells untreated with celastrol; ***P<0.0005 compared with ovalbumin-stimulated guinea pig mast cells untreated with celastrol. (C) The IgE-sensitized RBL2H3 cells, pretreated with or without celastrol (10 µM) for 15 min, were stimulated with DNP-HSA (100 ng/ml) for 1 h. Cells were dispersed into 24-well plates that had been coated with fibronectin (10 µg/cm² each) or 3 % BSA. Attached cells were stained with hematoxylin and eosin reagent and were counted under light microscopy. **P<0.0005 as compared with RBL2H3 cells without antigen stimulation; +P<0.05 as compared with antigen stimulated RBL2H3 cells untreated with celastrol; **P<0.005 as compared with antigen-stimulated RBL2H3 cells dispersed into 24-well plates that had been coated with fibronectin.
Celastrol remained in the binding site throughout a 5 ns molecular dynamics simulation, demonstrating the stability of the ERK2-celastrol complex (data not shown). Celastrol binds to a large pocket in ERK2 (Fig. 3A), which serves as the ATP-binding site. Many drugs have also been designed to target the same site (Aronov et al., 2007). The carbonyl oxygen of celastrol forms a hydrogen bond with an N
proton of Lys52 and the hydroxy group of Tyr34 (Fig. 3B). Lys52 has been previously suggested to form a hydrogen bond with a pyrazolylpyrrole drug (Aronov et al., 2007). An additional hydrogen bond is formed between the carboxy oxygen of celastrol and the amide proton of Met106. The hydrocarbon skeleton of celastrol interacts hydrophobically with the alkyl side chains of nearby Val47 and Leu154. These hydrogen bonding and hydrophobic interactions largely contribute to the specificity and stability of the celastrol binding to ERK2.

These results suggest that celastrol may potentially bind to ERK2. However, more studies should be performed to confirm binding.

3.4. Celastrol exerts inhibitory effects on FcεRI signaling in antigen-stimulated RBL2H3 cells

Since celastrol specifically inhibited phosphorylation of ERK2, we hypothesized that celastrol affects FcεRI signaling. The effect of celastrol on FcεRI signaling was investigated. Antigen stimulation induced an interaction between FcεRIγ and ERK and an interaction between FcεRIγ and PKCδ (Fig. 4A). Treatment of cells with celastrol inhibited these interactions (Fig. 4A). Neither Akt nor Lyn showed an interaction with FcεRIγ (Fig. 4A). Rac1 was shown to interact with ERK and PKCδ (Fig. 4B). The interaction between FcεRIγ and ERK, and FcεRIγ and PKCδ was transient (Fig. 4A). ERK and PKCδ were found to interact with Rac1 (Fig. 4B), suggesting that Rac1 may be involved in FcεRIγ signaling. The inhibition of Rac1 exerted a negative effect on the phosphorylation of ERK by antigen (Fig. 4C), but not the phosphorylation of Akt. This finding suggests that Akt may act upstream of Rac1. The inhibition of ERK by PD98059 prevented the antigen from activating Rac1, confirming the interaction between Rac1 and ERK (Fig. 4D). The inhibition of ERK did not affect phosphorylation of Akt by antigen stimulation (Fig. 4D). This result suggests that Akt may function independently of ERK. These findings suggest that celastrol exerts anti-allergic effects by inhibiting FcεRI signaling involving interaction between Rac1 and ERK.

3.5. Celastrol inhibits PKCα and PKCδ to prevent the antigen from regulating the expression of the EMT-related proteins

We previously reported that antigen stimulation led to increased phosphorylation of PKCα and PKCδ (Kim et al., 2008). PKC signaling has been shown to be involved in EMT (Olmeda et al., 2007). Therefore, we hypothesized that antigen stimulation affects the expression of EMT-related proteins via PKC signaling. Treatment with celastrol inhibited the phosphorylation of PKCα and PKCδ in antigen-stimulated RBL2H3 cells and prevented the antigen from regulating the expressions of EMT-related proteins (Fig. 5A). For example, celastrol prevented the antigen from decreasing the expression of E-cadherin. Celastrol prevented the antigen from increasing the expression of N-cadherin, vimentin, and snail. Inhibition of PKCα by 12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile, 5,6,7,13-tetrahydro-13-methyl-5-oxo (Go6976) (1 µM) and inhibition of PKCδ by rottlerin (10 µM) prevented the antigen from regulating the expression of the EMT-related proteins.
Fig. 5. Celastrol inhibits PKCα and PKCδ to prevent the antigen from regulating the expression of the EMT-related proteins. (A) IgE-sensitized RBL2H3 cells were pretreated with or without celastrol (2.5 µM) followed by stimulation with DNP-HSA (100 ng/ml) for various time intervals. Cell lysates prepared at each time point were subjected to Western blot analysis (upper panel). Cell lysates were also subjected to PKCδ activity assay (lower panel). Error bars represent the average values of three independent experiments. (B) IgE-sensitized RBL2H3 cells were pretreated with or without Go6976 (1 µM) or rottlerin (10 µM) followed by stimulation with DNP-HSA (100 ng/ml) for various time intervals. Cell lysates prepared at each time point were subjected to Western blot analysis.

3.6. Celastrol inhibits Rac1 to prevent the antigen from regulating the expression of the EMT-related proteins

PKC signaling has been shown to activate Rac1 in various mammalian cells (Slupsky et al., 2007). PKC signaling is necessary for the regulation of the expression of the EMT-related proteins (Dissanayake et al., 2007). Since celastrol inhibited PKCα and PKCδ, we examined whether celastrol affected Rac1 activity. Treatment with celastrol inhibited Rac1 activity in antigen-stimulated RBL2H3 cells (Fig. 6A). Antigen stimulation increased the phosphorylation of rhoGDI (Fig. 6A). Increased phosphorylation of rhoGDI is known to be associated with the activation of Rac1 (Slupsky et al., 2007). Treatment with celastrol prevented the antigen from increasing phosphorylation of rhoGDI (Fig. 6A). Inhibition of Rac1 by a dominant negative construct prevented the antigen from regulating the expression of the EMT-related proteins (Fig. 6B). A Rac1 constitutive active mutant affected the expression of the EMT-related proteins (Fig. 6C), suggesting that Rac1 mediates the effect of antigen on the expression of these proteins. These results suggest that celastrol inhibits Rac1 activity to exert its effect on the expression of EMT-related proteins.

3.7. Celastrol exerts a negative effect on FcεRIβ signaling by inhibiting the interaction between hsp90 and Akt, PKCδ, and FcεRIβ

Since celastrol inhibits FcεRIγ signaling (Fig. 4A), it was necessary to examine whether celastrol exerted a negative effect on FcεRIβ signaling. Antigen stimulation induced an interaction between FcεRIβ and Akt and an interaction between FcεRIβ and Lyn (Fig. 7A). It is apparent that Rac1, ERK, and PKCα are not involved in FcεRIβ signaling (Fig. 7A). It has been shown previously that celastrol binds to heat shock protein 90 (hsp 90) (Zhang et al., 2008; Basso et al., 2002). Celastrol binding to hsp90 has been shown to inhibit the interaction between hsp90 and CDC37 (Zhang et al., 2008). HSP90 showed an initial interaction with FcεRIβ and Akt, followed by an interaction with PKCδ (Fig. 7B). Celastrol inhibited the interaction between hsp90 and FcεRIβ as well as the interaction between hsp90 and Akt and the interaction between hsp90 and PKCδ (Fig. 7B). As hsp90 showed an interaction with PKCδ, it is suggested that hsp90 is also involved in FcεRIγ signaling. These results indicate that celastrol binds to hsp90 to exert a negative effect on FcεRIβ signaling. PKCα did not show an interaction with FcεRIγ signaling. FcεRIγ signaling is mediated by elevated levels of serum IgE as a result of the application of dinitrofluorobenzene (DNFB) (Matsuda et al., 1997; Tomimori et al., 2005). Based on the finding that celastrol decreased the phosphorylation of ERK, as well as the secretion of β-hexosaminidase.

3.8. Celastrol exerts an in vivo anti-allergic effect

The in vivo anti-allergic effect of celastrol was investigated using Nc/Nga mice. Nc/Nga mice develop human atopic dermatitis (AD)-like lesions mediated by elevated levels of serum IgE.
and histamine release, we hypothesized that celastrol exerts an in vivo anti-allergic effect. Histology analysis showed increased skin inflammation following the exposure to DNFB, as expected (Fig. 8A). Skin inflammation induced by DNFB was reduced by the inhibition of ERK by PD98059 (Fig. 8A), suggesting that ERK mediates the allergic inflammation caused by exposure to DNFB (Fig. 8A). Treatment with celastrol significantly reduced skin inflammation (Fig. 9A). Topical application of DNFB to the ears also induced inflammation, and treatment with celastrol reduced the inflammation caused by DNFB (Fig. 8B). Ear thickness in Nc/Nga mice was determined. An application of DNFB to the ears once a week increased ear thickness (Fig. 8C), and celastrol prevented DNFB from increasing the ear thickness (Fig. 8C). We also examined whether celastrol would exert an in vivo anti-allergic effect in a 12-O-tetradecanoylphorbol-13-acetate (PMA)-induced skin inflammation model employing Balb/c mice. Treatment with celastrol effectively prevented PMA from inducing the secretion of Th2 cytokines.

Fig. 6. Celastrol decreases Rac1 activity to prevent the antigen from regulating the expression of the EMT-related proteins. (A) IgE-sensitized RBL2H3 cells were pretreated with or without celastrol (2.5 µM) for 15 min followed by stimulation with DNP-HSA (100 ng/ml) for various time intervals. Cell lysates prepared at each time point were subjected to Western blot analysis. (B) RBL2H3 cells were transiently transfected with a control vector or a Rac1 dominant negative construct (Rac1N17). The next day, RBL2H3 cells were sensitized for 16 h with DNP-specific IgE (100 ng/ml), and stimulated with DNP-HSA (100 ng/ml) for various time intervals. Cell lysates prepared at each time point were subjected to Western blot analysis. (C) RBL2H3 cells were transiently transfected with a control vector or cells were transfected with various amounts of a Rac1 constitutive active mutant construct (Rac1V12). At 48 h after transfection, cell lysates were prepared and subjected to Western blot analysis.

Fig. 7. Celastrol exerts a negative effect on FcεRI signaling by inhibiting the interaction between hsp90 and FcεRIβ, the interaction between hsp90 and Akt and the interaction between hsp90 and PKCd. (A) IgE-sensitized RBL2H3 cells were pretreated with or without celastrol (10 µM) for 15 min followed by stimulation with DNP-HSA (100 ng/ml) for various time intervals. Cell lysates prepared at each time point were immunoprecipitated with anti-FcεRIβ antibody (5 µg/ml). The immunoprecipitates were then subjected to Western blot analysis. (B) is the same as (A) except that Western blot analysis was performed.
such as IL-5 (Fig. 9A) and IL-13 (Fig. 9B). Celastrol prevented PMA from inducing secretion of IL-5 (Fig. 9C) and IL-13 (Fig. 9D) in concanavalin A (concanavalin A)-stimulated splenocytes. Celastrol exerted a negative effect on ear thickness (Fig. 9E) and skin inflammation (Fig. 9F).

4. Discussion

RBL2H3 cells are mucosal mast cells that express immunoglobulin Fc epsilon receptor I (FcεRI). Stimulation of IgE-sensitized RBL2H3 cells with a specific antigen triggers a cascade of events leading to degranulation, mediator release, activation of mitogen activated protein kinase (MAPK), tyrosine kinase, and phospholipase C, an increase in reactive oxygen species (ROS) production, calcium influx, and cytokine production (Reth, 1989; Cambier, 1995). P95 vav associates with FcεRIγ and p95 vav immunoprecipitates have identified the presence of Grb2, Raf-1, and ERK2 (Song et al., 1996). Tyrosine phosphorylation of vav links FcεRI to the Rac1 pathway (Teramoto et al., 1997). Rac1 mediates the effect of FcεRI on calcium mobilization and degranulation (Hong-Geller et al., 2001). FcεRI activation activates PKCδ, which activates ERK and induces degranulation (Liu et al., 2001). Activation of MAPK signaling has been shown to result in degranulation of eosinophils (Kampen et al., 2000). Inhibition of ERK causes an anti-inflammatory effect (Kim et al., 2007; Duan et al., 2004). Antioxidants have been shown to inhibit allergic inflammation, suggesting a role of reactive oxygen species (ROS) in allergic inflammation (Dharajiya et al., 2007). Celastrol has been shown to induce antioxidant responsive genes (Trott et al., 2008), suggesting an anti-inflammatory role of celastrol. Immunoprecipitation studies have shown that an interaction occurs between Rac1 and ERK (Fig. 4B). Rac1 and ERK cross regulate each other (Fig. 4C–D). Celastrol may inhibit Rac1 activity by binding to ERK. It has been shown previously that MAPK interacts with Rac1 (Vaidya et al., 2005). An interaction between Rac1 and ERK in antigen-stimulated RBL2H3 cells has not been reported. PKCδ has been shown to regulate ERK in FcεRI signaling to leukotriene synthesis in mast cells (Cho et al., 2004). Inhibition of PKCδ by Go696 and inhibition of PKCα by rottlerin have been shown to exert a negative effect on the phosphorylation of ERK (Kim et al., 2008). Inhibition of ERK exerts a negative effect on the phosphorylation of PKCα and PKCδ (unpublished observations). Antigen stimulation induces an interaction between PKCδ and ERK, but not an interaction between PKCα and ERK (unpublished observation). Celastrol inhibited the interaction between PKCδ and ERK (unpublished observations). This finding is understandable as celastrol binds to ERK2 (Fig. 3A–B). The PKC pathway is known to regulate the expression of the EMT-related proteins, such as N-cadherin (Delannoy et al., 2001). Vimentin is known to be secreted in activated macrophages and acts as substrate for PKC (Mor-Vaknin et al., 2003). Celastrol treatment inhibited Rac1 activity in antigen-stimulated RBL2H3 cells (Fig. 6C). Rac1 and ROS are known to be involved in the EMT (Radisky et al., 2005). The EMT is known to play an important role in various cellular processes, including embryogenesis and tumorigenesis (Barberà et al., 2004). However, the role of these proteins in allergic inflammation remains unknown. A knock out of IL-4 has been shown to inhibit allergic airway inflammation (Nath et al., 2007), suggesting the role of the Th2 cytokine in allergic inflammation. Downregulation of E-cadherin has been shown to promote Th2 cell
activity (Heijink et al., 2007). It would be of interest to examine whether downregulation of E-cadherin would increase the level of Th2 cytokines such as IL-5 and IL-13 in antigen-stimulated RBL2H3 cells. Snail has been known to be critical for the EMT (Olmeda et al., 2007). The relationship between the EMT and allergic inflammation has not been studied extensively. As celastrol prevented the antigen from regulating the expression of EMT-related proteins, we hypothesized that EMT-related proteins have a role in allergic inflammation. Downregulation of the snail gene prevented the antigen from regulating the expression of the EMT-related proteins (data not shown). Downregulation of snail did not affect phosphorylation of PKCα or PKCδ (data not shown), confirming that PKC functions upstream of snail. Downregulation of snail exerted a negative effect on the secretion of IL-5 and IL-13 in antigen-stimulated RBL2H3 cells (data not shown). Inhibition of MMP-2 by GM 6001 exerted the same effect as the downregulation of snail on the secretion of IL-5 and IL-13 (data not shown). These results indicate that the EMT is closely related with allergic inflammation. Inhibition of MMP-2 by GM6001 exerted a negative effect on the secretion of β-hexosaminidase in antigen-stimulated RBL2H3 cells (data not shown). MMP-2 has been shown to play a role in allergic inflammation (Kumagai et al., 1999). These findings confirm the role of MMP-2 in allergic inflammation. The effect of snail on secretion of Th2 cytokines have not been reported for RBL2H3 cells. The induction of Th2 cytokine such as GM-CSF has been shown to occur via ERK (Kawaguchi et al., 2004). ICAM-1-induced expression of proinflammatory cytokines has been shown to require ERK (Lee et al., 2000). We found that the inhibition of ERK prevented the antigen from inducing secretion of IL-5 and IL-13 (data not shown).

Based on the above results, it is apparent that celastrol regulates the expression of the EMT-related proteins though inhibition of FcεRI signaling, involving PKC, Rac1 and ERK. The role of hsp90 in allergic inflammation has been reported (Mayor et al., 2007; Chatterjee et al., 2007). Celastrol inhibits Akt activity by interrupting the interaction between hsp90 and CDC37 (Gray et al.,

Fig. 9. Celastrol inhibits skin inflammation induced by PMA in Balb/c mice. (A) Celastrol decreases the serum level of IL-5. **P<0.005 as compared to Balb/c mice untreated with PMA; ++ P<0.005 as compared with Balb/c mice without celastrol treatment. (B) is the same as (A) except that serum level of IL-13 was measured. *P<0.05 as compared with Balb/c mice untreated with PMA; +++ P<0.005 as compared with Balb/c mice without celastrol treatment. (C) Celastrol decreases IL-5 in concanavalin A-stimulated splenocytes. **P<0.005 as compared with splenocytes without Concanavalin A stimulation; + P<0.05 as compared with splenocytes without celastrol treatment. (D) Celastrol decreases IL-5 in concanavalin A-stimulated splenocytes. *P<0.05 as compared with splenocytes without Concanavalin A stimulation; ++ P<0.005 as compared with splenocytes without celastrol treatment. (E) The ear thickness of Nc/Nga mice treated with DNFB was measured 4 h after the application of PMA. **P<0.005 as compared to Balb/c mice without PMA treatment; +++ P<0.005 as compared with Balb/c mice without celastrol treatment. (F) Skin inflammation was induced by the topical application of PMA, along with or without celastrol to both ears. Skins were excised at four weeks and the skin sections were stained with hematoxylin and eosin.
Downregulation of CDC37 has been shown to reduce the activity of ERK and Akt (Gray et al., 2007). Molecular dynamics simulation showed the binding of ceruloplasmin to hsp90 (Zhang et al., 2008). The role of Akt in allergic airway inflammation has been reported (Lee et al., 2006; Pinho et al., 2005). Inhibition of Akt prevented the antigen from regulating the expression of the EMT-related protein in RBL2H3 cells (data not shown). Inhibition of Akt did not affect the phosphorylation of ERK or Rac1 activity in antigen-stimulated RBL2H3 cells (data not shown), suggesting that Akt and ERK employ different signaling pathways to exert an effect on allergic inflammation. Based on the finding that hsp90 binds to Akt (Fig. 7B), it is apparent that ceruloplasmin inhibits Akt through binding to hsp90. Previously, we reported that antigen stimulation induced an interaction between CD44 and PKCα (Kim et al., 2008). HSP90 was shown to interact with PKCα in response to antigen stimulation (Fig. 7B). Ceruloplasmin decreased the interaction between hsp90 and PKCα (Fig. 7B). Therefore, it is likely that ceruloplasmin inhibits PKCα through binding to hsp90. HSP90 binds to PKCδ, suggesting a role in FcεRI signaling (Fig. 7B). This result suggests that hsp90 may involve in both FcεRI and FcγRI signaling.

Based on the results we obtained in this study, we propose the following mechanism of the anti-allergic effect caused by ceruloplasmin. Ceruloplasmin inhibits FcεRI signaling through its binding to ERK. Ceruloplasmin inhibits FcγRI signaling by inhibiting the interaction between FcγRI and Akt and by inhibiting the interaction between FcεRI and Lyn. It is apparent that hsp90 plays an important role in FcεRI signaling through its binding to FcγRI and Akt. In this study, we established a relationship between the EMT and allergic inflammation, and further dissected FcεRI signaling. Application of ceruloplasmin for various in vivo atopic dermatitis animal models would be necessary to enhance the clinical value of ceruloplasmin.

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Basso, A.D., Solit, D.B., Chiosis, G., Bird, S., Tsichlis, P., Rosen, N., 2002. Akt forms an apparent that hsp90 plays an important role in FcεRI signaling (Fig. 7B). This result suggests that Akt and ERK employ different signaling pathways to exert an effect on allergic inflammation. Based on the finding that hsp90 binds to Akt (Fig. 7B), it is apparent that ceruloplasmin inhibits Akt through binding to hsp90. Previously, we reported that antigen stimulation induced an interaction between CD44 and PKCα (Kim et al., 2008). HSP90 was shown to interact with PKCα in response to antigen stimulation (Fig. 7B). Ceruloplasmin decreased the interaction between hsp90 and PKCα (Fig. 7B). Therefore, it is likely that ceruloplasmin inhibits PKCα through binding to hsp90. HSP90 binds to PKCδ, suggesting a role in FcεRI signaling (Fig. 7B). This result suggests that hsp90 may involve in both FcεRI and FcγRI signaling.

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