

Celastrol suppresses expression of adhesion molecules and chemokines by inhibiting JNK-STAT1/NF- κ B activation in poly(I:C)-stimulated astrocytes

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In the central nervous system, viral infection can induce inflammation by up-regulating pro-inflammatory mediators that contribute to enhanced infiltration of immune cells into the central nervous areas. Celastrol is known to exert various regulatory functions, including anti-microbial activities. In this study, we investigated the regulatory effects and the mechanisms of action of celastrol against astrocytes activated with polyinosinic-polycytidylic acid (poly(I:C)), a synthetic dsRNA, as a model of pro-inflammatory mediated responses. Celastrol significantly inhibited poly(I:C)-induced expression of adhesion molecules, such as ICAM-1/VCAM-1, and chemokines, such as CCL2, CXCL8, and CXCL10, in CRT-MG human astrogloma cells. In addition, celastrol significantly suppressed poly(I:C)-induced activation of JNK MAPK and STAT1 signaling pathways. Furthermore, celastrol significantly suppressed poly(I:C)-induced activation of the NF- κ B signaling pathway. These results suggest that celastrol may exert its regulatory activity by inhibiting poly(I:C)-induced expression of pro-inflammatory mediators by suppressing activation of JNK MAPK-STAT1/NF- κ B in astrocytes. [BMB Reports 2017; 50(1): 25-30]

INTRODUCTION

Viral infection induces neuro-inflammation that contributes to the development of viral encephalitis and various neurodegenerative conditions (1, 2). In the central nervous system (CNS), astrocytes play an important role in virus-induced neuro-inflammation (3). Upon viral infection, astrocytes produce various pro-inflammatory mediators such as adhesion molecules, and chemokines, which participate in the recruitment of

immune cells into the central nervous areas (4-6).

Viral infection results in release of double-stranded RNA (dsRNA) which is a viral genome or an intermediate during viral replication. Toll-like receptor-3 (TLR3) recognizes the dsRNA and activates intracellular signaling pathways, involving mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription 1 (STAT1), and nuclear factor kappa B (NF- κ B), resulting in inflammatory responses (2, 6, 7).

Upon viral infection or stimulation with dsRNA including polyinosinic-polycytidylic acid (poly(I:C)), a synthetic dsRNA, astrocytes known to preferentially express TLR3 are activated via TLR3 (4, 8-10). The activated astrocytes then express various pro-inflammatory mediators such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), CC chemokine ligand 2 (CCL2; monocyte chemoattractant protein-1), CXC chemokine ligand 8 (CXCL8; IL-8) and CXCL10 (interferon-gamma-induced protein-10) (9, 10).

Celastrol is a quinone methide triterpenoid derived from *Tripterygium wilfordii* Hook (11). Several reports suggest that celastrol possesses anti-microbial and anti-inflammatory activities in various experimental models (12-16). Celastrol has been shown to suppress expression of lipopolysaccharide (LPS)-induced pro-inflammatory cytokines; these cytokines are produced through MAPK signal transduction and NF- κ B in microglial cells (13). Celastrol also inhibits HIV-1 Tat transactivation function by covalently modifying the cysteine thiols (15). Celastrol inhibits HIV-1 Tat-induced pro-inflammatory responses by blocking the JNK MAPK-AP-1/NF- κ B signaling pathways and inducing HO-1 expression in astrocytes (16). In microglia, although the molecular mechanisms underlying its action were not determined, celastrol was reported to suppress poly(I:C)-induced expression of pro-inflammatory cytokines and chemokines (14). Taken together, these studies suggest that celastrol may exert anti-microbial and inflammatory effects by modulating function of viral and cellular target proteins.

In this work, we investigated the inhibitory effects and the mechanisms of action of celastrol on poly(I:C)-induced expression of pro-inflammatory mediators, such as adhesion molecules and chemokines, in CRT-MG human astrogloma

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cells. Celastrol significantly suppressed poly(I:C)-induced expression of ICAM-1/VCAM-1 adhesion molecules, and CCL2, CXCL8 and CXCL10 chemokines. Celastrol inhibited the signaling pathways leading to JNK-STAT1 activation as well. We also observed that celastrol suppressed poly(I:C)-induced signaling cascades that lead to NF- κ B activation. Our findings show that celastrol inhibits poly(I:C)-induced expression of pro-inflammatory mediators by suppressing activation of JNK MAPK-STAT1/NF- κ B in astrocytes.

RESULTS

Celastrol inhibits poly(I:C)-induced expression of adhesion molecules and chemokines in CRT-MG cells

The experiment with poly(I:C) stimulation has been used as an *in vitro* model for virus infection. Stimulation of astrocytes with poly(I:C) has been shown to induce pro-inflammatory mediators, such as CCL2, CXCL8, and CXCL10 (4-6). Since adhesion molecules and chemokines play important roles in recruitment of immune cells from circulation to the site of inflammation in the CNS, we investigated the effects of celastrol on poly(I:C)-induced expression of ICAM-1/VCAM-1

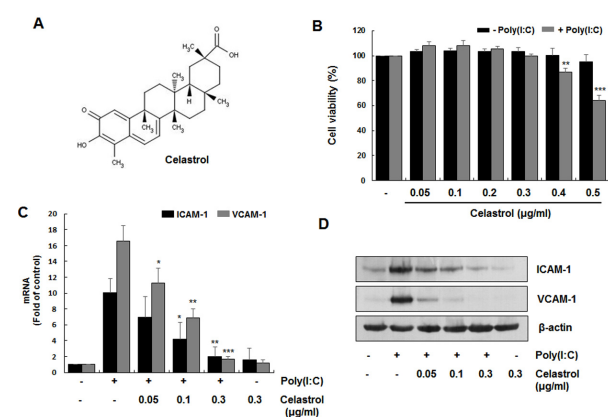


Fig. 1. Celastrol inhibits poly(I:C)-induced expression of adhesion molecules in CRT-MG cells. (A) The chemical structure of celastrol is shown. (B) To evaluate the cytotoxic effects of celastrol, CRT-MG cells were incubated with various concentrations of celastrol in the absence or presence of poly(I:C) (5 μ g/ml) for 24 h and cell viability was assessed by the MTT assay. ** $P < 0.01$ and *** $P < 0.001$ compared to the control cells. (C) Inhibitory effects of celastrol on poly(I:C)-induced mRNA expression of ICAM-1/VCAM-1. CRT-MG cells were pretreated with celastrol (0.05, 0.1, 0.3 μ g/ml) for 1 h, and then stimulated with poly(I:C) (5 μ g/ml) for 3 h. The levels of ICAM-1/VCAM-1 mRNA were assessed by quantitative RT-PCR. Data are presented as mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with the cells stimulated with poly(I:C) alone. (D) Inhibitory effects of celastrol on poly(I:C)-induced protein expression of ICAM-1/VCAM-1. CRT-MG cells pretreated with celastrol for 1 h were stimulated with poly(I:C) (5 μ g/ml) for 12 h. The levels of ICAM-1/VCAM-1 protein were determined by Western blot analysis.

adhesion molecules, and CCL2, CXCL8 and CXCL10 chemokines in CRT-MG human astroglia cells. We first evaluated the effect of celastrol (Fig. 1A) in the presence or absence of poly(I:C) on viability of CRT-MG cells using MTT assay. As shown in Fig. 1B, celastrol did not show any cytotoxicity at concentrations of up to 0.3 μ g/ml. CRT-MG cells were also pretreated with various concentrations of celastrol, and stimulated with poly(I:C), and expression levels of ICAM-1/VCAM-1 adhesion molecules were measured by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses. Celastrol markedly inhibited poly(I:C)-induced mRNA and protein expression of ICAM-1/VCAM-1 (Fig. 1C and D). We further analyzed the effect of celastrol on the expression of CCL2, CXCL8 and CXCL10 chemokines in poly(I:C)-stimulated CRT-MG cells. As shown in Fig. 2A, celastrol suppressed poly(I:C)-induced CCL2, CXCL8 and CXCL10 mRNA expression in a dose-dependent manner. Consistent with the mRNA level changes, celastrol significantly decreased poly(I:C)-induced production of CCL2, CXCL8 and CXCL10 in the culture media, as determined by enzyme-linked immunosorbent assay (ELISA) (Fig. 2B).

Celastrol inhibits poly(I:C)-induced activation of JNK and STAT1 in CRT-MG cells

MAPKs, such as p38, c-Jun NH₂-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), have been impli-

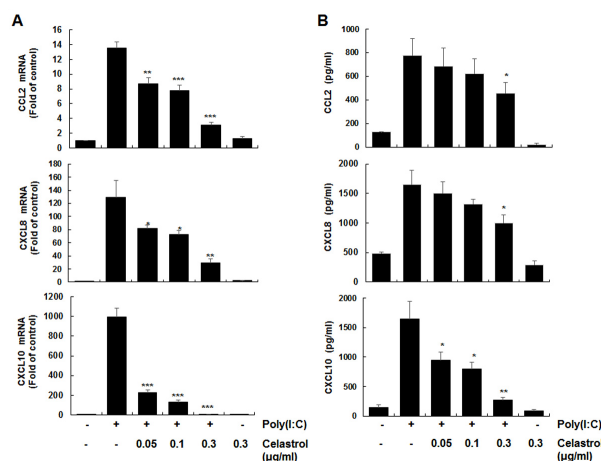


Fig. 2. Celastrol inhibits poly(I:C)-induced expression of chemokines in CRT-MG cells. (A) Cells were pretreated with celastrol for 1 h, and then stimulated with poly(I:C) (5 μ g/ml) for 3 h. Total RNA was prepared from cells and then analyzed for mRNA expression of CCL2, CXCL8, CXCL10, and β -actin by quantitative RT-PCR using specific primers. (B) Cells pretreated with celastrol for 1 h were stimulated with poly(I:C) (5 μ g/ml) for 24 h. Culture media of cells were collected and analyzed for production of CCL2, CXCL8, and CXCL10 by ELISA. Data are presented as mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with the cells stimulated with poly(I:C) alone.

cated in the expression of various pro-inflammatory mediators upon virus infection (6, 7). As such, we assessed the effect of celastrol on poly(I:C)-induced MAPK activation by Western blot analysis using phospho-specific antibodies. Poly(I:C) stimulation increased phosphorylation of all three MAPKs (Fig. 3A and B), and we found that celastrol inhibited poly(I:C)-induced phosphorylation of JNK in a dose-dependent manner (Fig. 3A and B). At the same time, celastrol was not able to reduce poly(I:C)-induced phosphorylation of ERK and p38, but it rather led to increased phosphorylation of ERK and p38 MAPKs in the treated cells. Previous studies reported JNK mediating poly(I:C)-induced activation of STAT1 (17, 18), with poly(I:C) stimulation, STAT1 transcription factor plays a significant role in the expression of various pro-inflammatory mediators in astrocytes (7, 8, 19). Therefore, we next investigated the effect of celastrol on poly(I:C)-induced STAT1 activation. We observed that celastrol inhibited poly(I:C)-induced phosphorylation of STAT1 in a dose-dependent manner (Fig. 3C). To explore the functional correlation between JNK activation and STAT1 activation, we used a pharmacological inhibitor of JNK. As shown in Fig. 3D, SP600125, a JNK

inhibitor, suppressed poly(I:C)-induced STAT1 activation in a dose-dependent manner, indicating the involvement of JNK MAPK in poly(I:C)-induced STAT1 activation. These results suggest that celastrol inhibits poly(I:C)-induced expression of adhesion molecules and chemokines via blocking JNK and STAT1 activation.

Celastrol inhibits poly(I:C)-induced signaling cascades leading to NF- κ B activation in CRT-MG cells

NF- κ B is another important transcription factor involved in the expression of various pro-inflammatory mediators, including chemokines (20). Poly(I:C) stimulation induces IKK activation, I κ B α degradation, and p65 phosphorylation, leading to the transcriptional activation of NF- κ B responsive genes (6). We next analyzed the effect of celastrol on the signaling cascades leading to NF- κ B activation. As shown in Fig. 4A and B, celastrol significantly suppressed poly(I:C)-induced IKK α / β phosphorylation, I κ B α degradation and p65 phosphorylation in a dose-dependent manner. Celastrol also inhibited poly(I:C)-induced p65 DNA-binding activity, as determined by EMSA

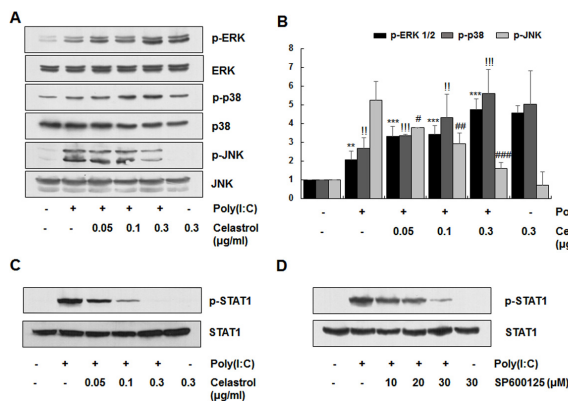


Fig. 3. Celastrol inhibits poly(I:C)-induced activation of JNK and STAT1 in CRT-MG cells. (A) Cells were pretreated with celastrol for 1 h, and then stimulated with poly(I:C) (5 μ g/ml) for 45 min. Whole cell extracts were prepared and analyzed for MAPK activation by Western blotting using specific antibodies. (B) Relative protein levels of each MAPK were quantified by scanning densitometry and normalized to control protein levels. Data are presented as mean \pm SD of three independent experiments. **P < 0.01, #P < 0.05, ***P < 0.001, and ###P < 0.001, compared with the control cells. #P < 0.05, ##P < 0.01, and ###P < 0.001, compared with the cells stimulated with poly(I:C) alone. (C) Cells were pretreated with celastrol for 1 h, and then stimulated with poly(I:C) for 3 h. Whole cell extracts were prepared and analyzed for STAT1 activation by Western blotting using specific antibodies. (D) Cells were pretreated with JNK inhibitor (SP600125) (10, 20, 30 μ M) for 1 h, and then stimulated with poly(I:C) for 3 h. Cell lysates were prepared and the levels of total and phosphorylated STAT1 were determined by Western blot analysis.

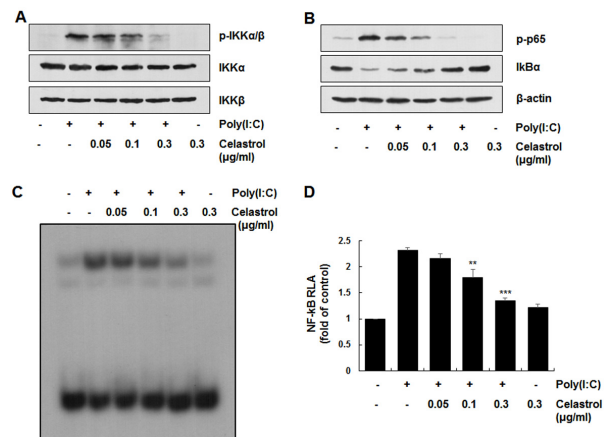


Fig. 4. Celastrol inhibits poly(I:C)-induced NF- κ B activation in CRT-MG cells. (A) Cells were pretreated with celastrol for 1 h, and then stimulated with poly(I:C) (5 μ g/ml) for 45 min. Whole cell extracts were prepared and analyzed for IKK α / β activation by Western blotting using phospho-specific antibodies. (B) The levels of total I κ B α and phosphorylated p65 in whole cell extracts were determined by Western blotting using phospho-specific antibodies. (C) Cells were pretreated with celastrol for 1 h and stimulated with poly(I:C) (5 μ g/ml) for 1 h. Nuclear extracts were prepared and analyzed for p65 DNA-binding activity by EMSA. (D) Cells were transiently transfected with a NF- κ B promoter-luciferase plasmid and a β -galactosidase plasmid (pCMV-lacZ). At 24 h after transfection, cells were treated with celastrol for 1 h, and followed by stimulation with poly(I:C) (5 μ g/ml) for 3 h, and then luciferase activity was determined. Luciferase activities were normalized to the β -galactosidase activity and expressed as fold increase over the control. RLA, relative luciferase activity. Data are presented as mean \pm SD of three independent experiments. **P < 0.01 and ***P < 0.001, compared with the cells stimulated with poly(I:C) alone.

(Fig. 4C). In addition, celastrol significantly decreased NF- κ B promoter activity in poly(I:C)-stimulated CRT-MG cells (Fig. 4D). These findings suggest that celastrol exerts its inhibitory effects on poly(I:C)-induced expression of pro-inflammatory mediators, such as ICAM-1/VCAM-1 adhesion molecules, and chemokines, such as CCL2, CXCL8 and CXCL10, via inhibition of NF- κ B signaling pathway.

DISCUSSION

Although inflammatory responses are a first line of host defense against viral infection, chronic and dysregulated inflammation in the CNS results in viral encephalitis and various neurodegenerative disorders (1, 2, 20). Viral dsRNA is released during viral replication cycle, and it is recognized by TLR3 on astrocytes, leading to activation of intracellular signaling pathways, such as MAPK, STAT1, and NF- κ B, and resulting in generation of immune responses in astrocytes (6, 7). Human astrocytes preferentially express TLR3, which mediates inflammatory responses upon dsRNA stimulation (4, 9, 10). The recruitment of immune cells to an inflammatory site in the CNS is an important step during the inflammatory response. Expression of adhesion molecules and cytokines/chemokines contributes to infiltration and retention of immune cells in the CNS. In this study, we demonstrated that celastrol inhibited poly(I:C)-induced expression of adhesion molecules, such as ICAM-1/VCAM-1 and chemokines, such as CCL2, CXCL8 and CXCL10, by suppressing activation of JNK MAPK-STAT1/NF- κ B in astrocytes.

Upon dsRNA stimulation such as with poly(I:C), astrocytes express various pro-inflammatory mediators, such as ICAM-1/VCAM-1 adhesion molecules, and CCL2, CXCL8, and CXCL10 chemokines, and these, in turn, contribute to infiltration of immune cells to the CNS (4, 8-10). Increased expression of CCL2, CXCL8 and CXCL10 has been reported to be associated with the development of neurodegenerative diseases, such as multiple sclerosis (MS), Parkinson's disease (PD), HIV-associated dementia, and Alzheimer's disease (AD) (21). Previous study reported that celastrol abrogated poly(I:C)-induced expression of pro-inflammatory cytokines and chemokines in microglia (14). Consistent with these results, we observed that celastrol significantly suppressed expression of ICAM-1/VCAM-1 adhesion molecules, and CCL2, CXCL8 and CXCL10 chemokines in poly(I:C)-stimulated CRT-MG cells. We previously reported that celastrol significantly decreased production of CCL2 but not CXCL8 and CXCL10 in cytokine-stimulated RINm5F pancreatic β -cells (12). This discrepancy may be due to the different effects of celastrol on specific pathways leading to chemokine expression in different cell types.

Since the MAPK signaling pathways play important roles in the expression of adhesion molecules and chemokines, blocking the MAPK activity is considered to be one of the strategies to modulate inflammatory diseases. Previous studies reported that MAPKs, including ERK, JNK and p38, were

involved in poly(I:C)-induced expression of pro-inflammatory mediators in astrocytes (7, 8). In agreement with those reports, we observed that stimulation of CRT-MG cells with poly(I:C) induced activation of all three MAPKs. Among these MAPKs, celastrol inhibited poly(I:C)-induced activation of JNK, but not ERK and p38 MAPKs. Instead, celastrol increased phosphorylation of ERK and p38 MAPKs.

Previous studies have demonstrated that JNK is involved in poly(I:C)-induced STAT1 activation (17, 18), and we found that celastrol suppressed poly(I:C)-induced activation of STAT1. This is of significance as STAT1 plays important roles in the expression of various pro-inflammatory mediators including cytokines (7). It was unclear whether JNK activation mediates poly(I:C)-induced STAT1 activation in astrocytes. Here, using a pharmacological inhibitor of JNK, it was demonstrated that JNK was also involved in poly(I:C)-induced STAT1 activation. These results suggest that celastrol exerts its inhibitory effects on poly(I:C)-induced expression of adhesion molecules and chemokines by suppressing JNK-STAT1 signaling pathways.

Stimulation with dsRNA activates the NF- κ B signaling pathway involved in the expression of various pro-inflammatory mediators (6). Sustained activation of NF- κ B contributes to the chronic inflammation, contributing to the development of neurodegenerative states. One study showed that celastrol inhibits IKK activity by directly targeting cysteine 179 in IKK (22). Consistent with this result, we observed that celastrol inhibited poly(I:C)-induced phosphorylation of IKK α / β . Celastrol also suppressed poly(I:C)-induced I κ B α degradation and p65 phosphorylation, which are downstream signaling mediators of IKK. In addition, celastrol inhibited poly(I:C)-induced DNA-binding activity of p65 NF- κ B. Furthermore, celastrol suppressed NF- κ B promoter activity in poly(I:C)-stimulated cells.

In conclusion, we provide evidence that celastrol exerts protective effect against poly(I:C)-induced expression of pro-inflammatory mediators by inhibiting the signaling cascades leading to activation of JNK MAPK-STAT1/NF- κ B in astrocytes. Elucidation of the action mechanisms by which celastrol inhibits poly(I:C)-induced inflammatory responses provides a molecular basis by which celastrol can be developed as a therapeutic agent against virally associated neuro-inflammation.

MATERIALS AND METHODS

Cell culture and reagents

CRT-MG human astroglial cells were maintained as described previously (16). Poly(I:C) was purchased from Sigma (St. Louis, MO, USA). MTT was obtained from Duchefa (Haarlem, the Netherlands). Primary antibodies against ICAM-1, VCAM-1, phosphor-p65, IKK α , IKK β and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and phosphor-p38, phosphor-ERK, or phosphor-JNK, total-p38 MAPK, total-ERK, total-JNK and phosphor-IKK α / β (Cell Signaling Technology, Beverly, MA, USA) were obtained commercially. Celastrol was purchased from Sigma, dissolved in dimethyl sulfoxide at 10 mg/ml, and

stored at -20°C .

MTT assay

Cytotoxic effects of celastrol were assessed using a MTT colorimetric assay as described previously (12).

Western blot analysis

Cells were collected and incubated with lysis buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 10% v/v glycerol) at 4°C for 30 min. Cell lysates were clarified by centrifugation. The amount of proteins in cell lysates was determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA). The protein samples (30–50 μg) were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel and were transferred onto a nitrocellulose membrane. The blots were blocked with 10% dry milk in TBST (137 mM NaCl, 20 mM Tris-HCl, pH 7.6, 0.1% Tween 20) and incubated with the indicated antibodies (1:1,000). The blots were then incubated with secondary antibodies (1:10,000) conjugated to horseradish peroxidase. The antibody-reactive protein bands were then detected by an enhanced chemiluminescence detection kit (Millipore, Billerica, MA, USA) (23).

RT-PCR analysis

Total RNA was extracted from cells using the Trizol reagent kit (Invitrogen, Gaithersburg, MD, USA). The RNA (2 μg) was reverse-transcribed with 10,000 U of reverse transcriptase and 0.5 $\mu\text{g}/\mu\text{l}$ oligo-(dT)₁₅ primer (Promega, Madison, WI, USA). The cDNA was amplified for quantitative PCR using the THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan) with the primer sets described previously (24). The PCR products were resolved on a 1% agarose gel and visualized with UV light after ethidium bromide staining.

Enzyme-linked immunosorbent assay (ELISA)

CRT-MG cells were pretreated with celastrol for 1 h and then stimulated with poly(I:C) for 24 h. The culture supernatants were then harvested and analyzed for production of CCL2, CXCL8, and CXCL10 chemokines by ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)

Cells were pretreated with celastrol for 1 h and then stimulated with poly(I:C) (5 $\mu\text{g}/\text{ml}$) for 1 h. Nuclear extracts were prepared from the cells and analyzed for NF- κB binding activity using an EMSA as described previously (25, 26).

Transfection

To perform the reporter assay, cells were co-transfected with NF- κB -luc reporter plasmid (Stratagene, La Jolla, CA, USA) and a control (pCMV- β -galactosidase) plasmid using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol (24). After a 48 h transfection, the cells were treated with celastrol for 1 h and then stimulated

with poly(I:C) for 24 h. Cell lysates were prepared and analyzed for luciferase and β -galactosidase activities. The luciferase activity of each sample was normalized to β -galactosidase activity to calculate the relative luciferase activity, and the results were expressed as fold transactivation.

Statistical analysis

Results are expressed as mean \pm standard error (SD) values from at least three independent experiments. The values were evaluated via one-way analysis of variance, followed by Duncan's multiple range test using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant at $P < 0.05$.

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CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

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