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Celastrol attenuates interleukin-8 release from LPS-activated human monocytes and monocyte-derived macrophages by inhibiting the NF-κB signalling pathway

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ABSTRACT: Interleukin-8 (IL-8), a potent neutrophil chemotactic factor, plays a critical role during inflammation. IL-8 is produced primarily by several immune cell types upon lipopolysaccharide (LPS) stimulation by activating the nuclear factor-κB (NF-κB) signalling pathway. NF-κB is an important transcription factor implicated in the inflammatory response, and the NF-κB signalling pathway is a potential target for inhibition by anti-inflammatory compounds. Celastrol has been shown to have therapeutic potential in treating many inflammatory diseases; however, there is little information on its ability to attenuate IL-8 production in human monocytes and macrophages. Here, we determined the effects of celastrol on LPS-induced IL-8 release and its molecular mechanism. Celastrol treatment significantly reduced IL-8 release from LPS-activated human monocytes and monocyte-derived macrophages with IC_{50} values of 3.13 ± 0.03 μM and 3.18 ± 0.05 μM, respectively. Additionally, the inhibitory effect of celastrol on the production of IL-8 was related to the modulation of IL-8 mRNA levels. Pre-treatment with celastrol significantly inhibited IKK, IκBα, and p65 phosphorylation and also prevented IκBα degradation due to LPS activation. Collectively, these results demonstrate that celastrol attenuates the release of IL-8 from LPS-activated human monocytes and monocyte-derived macrophages and suggest that inhibition of NF-κB signalling activation is likely a mechanism for the attenuation. This anti-inflammatory effect further highlights the therapeutic potential of celastrol.

KEYWORDS: celastrol, interleukin-8, NF-κB, monocytes, monocyte-derived macrophages

INTRODUCTION

The immune system's response to infections and tissue damages is characterized by the recruitment of immune cells such as neutrophils, monocytes, and macrophages, which results in inflammation [1]. Chemotactic signals guide immune cells to lesion sites; however, recruitment of an excessive number of immune cells causes extensive tissue damage and may result in chronic inflammation [2].

Interleukin-8 (IL-8) is a proinflammatory cytokine that stimulates the migration of neutrophils, monocytes, and macrophages [3]. IL-8 is produced by monocytes, macrophages, lymphocytes, fibroblasts, and endothelial cells after lipopolysaccharide (LPS) stimulation [4, 5]. Besides, IL-8 is considered the most potent chemoattractant for neutrophils, and it plays a critical role in systemic inflammation [6] by stimulating the migration and activity of neutrophils via CXCR1 and CXCR2 chemokine receptors [3].

Many cell-signalling events, that contribute to cytokine synthesis and release, respond to LPS exposure. LPS stimulates the production of IL-8 by both monocytes and macrophages via the nuclear factor-κB (NF-κB) signalling pathway [7]. The binding of LPS to toll-like receptor 4 (TLR4), which is expressed on monocytes and macrophages, activates the transcription factor NF-κB, which orchestrates the expression of inflammatory cytokine genes [8]. NF-κB is a critical transcription factor implicated in inflammation; thus, the NF-κB signalling pathway is a target of many inhibitors with significant therapeutic potential for treating inflammatory diseases and cancers [9, 10]. For example, anti-inflammatory corticosteroids (such as dexamethasone) inhibit IL-8 release and suppress expression of NF-κB [11]. However, clinically, the adverse side effects of corticosteroids are a concern.

Celastrol, a triterpene derivative originally identified in traditional Chinese medicine (Thunder God Vine), has shown therapeutic potential in several inflammatory diseases, including asthma, amyotrophic lateral sclerosis, and rheumatoid arthritis [12–14]. *In vitro* studies revealed the anti-inflammatory properties of celastrol in murine macrophages, microglia, and endothelial cells [15–17]. Anti-inflammatory activities of celastrol include the reduction of production of superoxide and myeloperoxidase and the release of elastase by human neutrophils [18]. Nonetheless, existing data regarding the ability of celastrol to attenuate IL-8 production in human monocytes and macrophages are limited. Therefore, this study was set out to determine whether celastrol inhibits IL-8 release by suppressing NF-κB signalling in LPS-activated human monocytes and monocyte-derived macrophages.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Sigma Aldrich, USA, unless stated otherwise. Celastrol and gliotoxin were purchased from TOCRIS Bioscience, USA. Iscove's Modified Dulbecco's modified medium (IMDM) was obtained from GIBCO, USA. DuoSet® enzyme-linked immunosorbent assay (ELISA) for human CXCL8/IL-8 was from R&D Systems®, USA. High Pure RNA Isolation Kit was obtained from Roche, Germany. SYBR® Safe DNA Gel Stain was from Invitrogen, USA.

Isolation and culture of human monocytes/monocyte-derived macrophages

This study was approved by the Ethical Review Board of the Faculty of Medicine, Thammasat University, Thailand (Certificate Numbers 069/2562 and 070/2562) and by the Institutional Biosafety Committee of Thammasat University (Allowance Numbers 027/2562 and 037/2562). Peripheral blood mononuclear cells were isolated by centrifugation using Ficoll-Hypaque centrifuge (density = 1.077, Robbins Scientific Corporation, USA), and monocytes were positively selected from the mononuclear cell fractions using $CD14^+$ immunomagnetic beads (Miltenyi Biotec, Germany) according to the manufacturer's instructions [19]. The monocytes were counted in an improved Neubauer chamber, and their viability was assessed by trypan blue exclusion. Based on flow cytometry of cells stained with anti-CD14 antibody, the obtained monocytes' purity was estimated at >98%. They were then cultured in IMDM containing 10% autologous serum and 100 IU/ml of penicillin and streptomycin for 7 days to allow differentiation of monocytes into monocyte-derived macrophages [20]. The medium was replaced after 3 days; non-adherent cells were discarded. Cultures were observed daily with an inverted phase microscope, and their purity was estimated at >98% based on the same method as the monocytes. Monocytes and monocyte-derived macrophages were resuspended, either in warm RPMI 1640 medium with 10% heat-inactivated foetal bovine serum for measurement of cell viability assay or in IMDM without phenol red, supplemented with 100 IU/ml of penicillin and streptomycin for measurement of IL-8 release and NF-κB activation after LPS treatment.

Cell viability assay

Cell viability was determined by colorimetric tetrazolium salt 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl-5-[(phenylamino) carboxy]-2H-tetrazolium hydroxide (XTT) assay. Cells (1×10^6 cells/ml) were incubated with 0.01–5 μM celastrol, 1 μM dexamethasone, and vehicle (0.2% DMSO) or RPMI 1640 (medium control) for 24 h at 37 °C. Then, XTT was added, and the mixture was incubated for 4 h at 37 °C. Cell viability was evaluated at 450 nm using a BioTex microplate reader with Gen 5 software [21]. Each assay was done in triplicate, and cell viability was calculated using the following equation:

$$\%\,\text{Viability} = \frac{(\text{OD sample} - \text{OD drug control}) \times 100}{(\text{OD cell control} - \text{OD medium control})}.$$

Controls were medium only (medium control), drugs with medium (drugs control), or cells with medium (cell control).

In vitro culture of monocytes and monocyte-derived macrophages for IL-8 assays

Firstly, we performed a kinetic study of IL-8 production from LPS-activated monocytes and monocyte-derived macrophages to determine the optimum time for LPS activation. Monocytes and monocyte-derived macrophages (2×10^6 cells/ml) were incubated with 10 ng/ml LPS for 1, 2, 4, 8, 10, 12, and 14 h. After incubation, supernatants were assayed for IL-8 in triplicate, and the values were averaged. Secondly, monocytes and monocyte-derived macrophages (2×10^6 cells/ml) were incubated with 0.5–5 μM celastrol for 24 h, with DMSO as a vehicle control or 1 μM dexamethasone as a positive control; and, then, treated or not treated with 10 ng/ml LPS for an additional 12 h [4]. Total RNA was extracted from these cells, and supernatants were collected for IL-8 ELISA assays, which were done according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

IL-8 in supernatants was quantified using the Human IL-8/CXCL8 DuoSet ELISA kit. Samples collected from cells treated with LPS were diluted 1/25 before analysis. The absorbance was measured on a Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, USA) using SkanIT 4.0 software at 450 nm with a wavelength correction of 570 nm. The inhibition of CXCL8/IL-8 production in LPS-treated cells by drugs in percentage was calculated using the following equation:

$$\%\ \text{Inhibition} = 100 - \frac{(\text{IL-8 conc. of drugs \& LPS-treated group})}{(\text{IL-8 conc. of LPS-treated group})} \times 100.$$

RNA extraction and RT-PCR

Total RNA was isolated from cell pellets using the High Pure RNA Isolation Kit. The cDNA was synthesized from 500 ng of total RNA using the HSRT 100 kit (Sigma) [22]. For each sample, 1 μl of 500 μM of each dNTP and 1 μl of 2.5 μM random nonamers were added and then incubated at 70 °C for 10 min. After incubation, 2 μl of 10× buffer for AMV-RT, 1 μl of 1 U/μl RNase inhibitor, and 1 μl of 1 U/μl enhanced

avian reverse transcriptase (RT) were added and incubated again at 45 °C for 50 min, followed by placing the sample on ice for subsequent PCR amplification. PCR analysis was carried out in a volume of 50 µl containing 1 µl of 200 µM of each dNTP, 5 µl of template DNA (cDNA) from the RT reaction, 1 µl of JumpStart AccuTaq LA DNA polymerase mix, and 1 µl of PCR primers. The human IL-8 forward primer was 5′-ATTTCTGCAGCTCTGTGTGAA-3′, and the reverse primer was 5′-TGAATTCTCAGCCCTCTTCAA-3′. The forward primer for human β-actin (as the internal standard) was 5′-TGCGTGACATTAAGGAGAAG-3′, and the reverse primer was 5′-CTGCATCCTGTCGGCAATG-3′. PCR amplification was performed using a thermocycler (T100; Bio-Rad, USA). The amplification for IL-8 was done with an initial activation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min. The amplification for actin was done with an initial activation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 68 °C for 1 min. The PCR product size of IL-8 and β-actin were 255 bp and 316 bp, respectively. The PCR products were separated on 2% agarose gels and visualized using SYBR® Safe DNA Gel staining. The gel images were captured on a gel documentation system (GelDoc Go, Bio-rad), and the yield of PCR product was normalized using the gene for actin as an internal control.

Western blot analysis

Cells (4×10^6 cells/ml) were incubated with 0.5, 1, or 5 µM celastrol, with 0.2% DMSO as a vehicle control or 1 µM gliotoxin (NF-κB inhibitor) as a positive control, for 24 h; and then activated with 10 ng/ml LPS for 45 min [20]. Total protein was extracted using RIPA buffer solution (Thermo Fisher Scientific), and the protein concentration was quantitated using Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific). An equal amount of protein (15 µg) from whole-cell lysates was used for SDS-PAGE. Proteins were separated on 4–12% NuPAGE Bis-Tris acrylamide and then transferred onto Invitrolon PVDF membranes (Thermo Fisher Scientific). After blocking with 5% skim milk for 1 h, the membranes were incubated at 4 °C overnight with the following primary antibodies: anti-p-IKKα (1:1,000; ab38515), anti-IKKα (1:1,000; ab32041), anti-p-IκBα (1:1,000; ab133462), anti-IκBα (1:1,000; ab32518), anti-p-NF-κB p65 (1:1,000; ab86299), anti-NF-κB p65 (1:1,000; ab16502), or anti-beta actin (1:1,000; ab8227). This was followed by incubation with the horseradish peroxidase-conjugated secondary antibody, anti-rabbit IgG H&L (1:2,000; ab205718), at room temperature for 1 h (all antibodies were from Abcam, Cambridge, USA). Target bands and band intensities were visualized using an enhanced chemiluminescence kit (ab65623, Abcam) and Odyssey infrared imaging system (ODYSSEY Fc station, LI-COR) with Image Studio software, respectively. β-actin was used as the internal loading control.

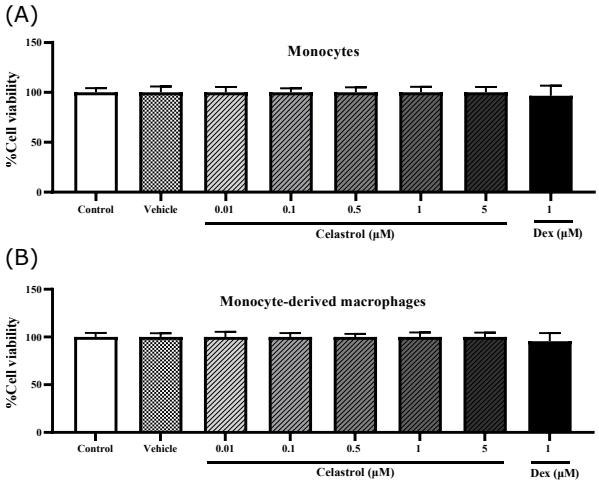

Fig. 1 Effects of 0.01–5 µM celastrol, 0.2% DMSO as vehicle control, and 1 µM dexamethasone (Dex) as a positive control on human monocytes (A) and monocyte-derived macrophages (B). Cells were treated with various concentrations of drugs for 24 h. The cell viability was determined using the XTT assay. Each bar represents the mean ± SD ($n = 4$).

Statistical analysis

Results were reported as mean ± standard deviation (SD). Statistical significance ($p < 0.05$) was determined by one-way ANOVA followed by Dunnett's t-test for multiple comparisons using GraphPad Prism 9.4.1 (GraphPad Prism, CA).

RESULTS

Effects of celastrol on cell viability

Cells were treated with various concentrations of celastrol for 24 h to determine the effects of celastrol on viability. Treatment of monocytes or monocyte-derived macrophages with 0.01–5 µM celastrol and 1 µM dexamethasone or with dexamethasone alone did not affect cell viability (Fig. 1A, 1B).

Kinetics of IL-8 production by LPS-activated monocytes and monocyte-derived macrophages

We measured the kinetics of IL-8 release in cells incubated with 10 ng/ml LPS for 1, 2, 4, 8, 10, 12, and 14 h to determine the optimum incubation time for LPS activation. Little IL-8 was secreted during the first 1–4 h; however, IL-8 levels increased rapidly after 6 h, and the maximum concentration was observed after 12 h in both monocytes (Fig. 2A) and monocyte-derived macrophages (Fig. 2B). Therefore, we used 12 h for LPS activation in this study.

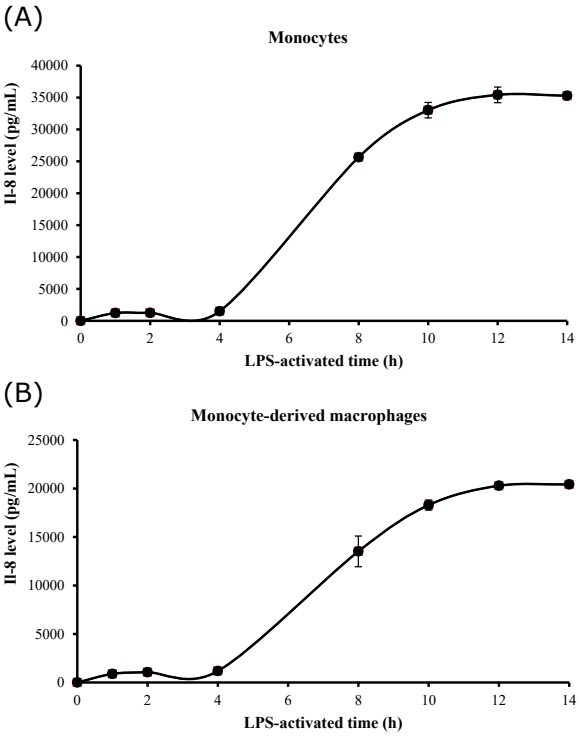

Fig. 2 Kinetics of IL-8 release by LPS-activated monocytes (A) and LPS-activated monocyte-derived macrophages (B). Cells were activated with 10 ng/ml LPS for 1, 2, 4, 8, 10, 12, and 14 h. Data are reported as mean ± SD ($n = 3$).

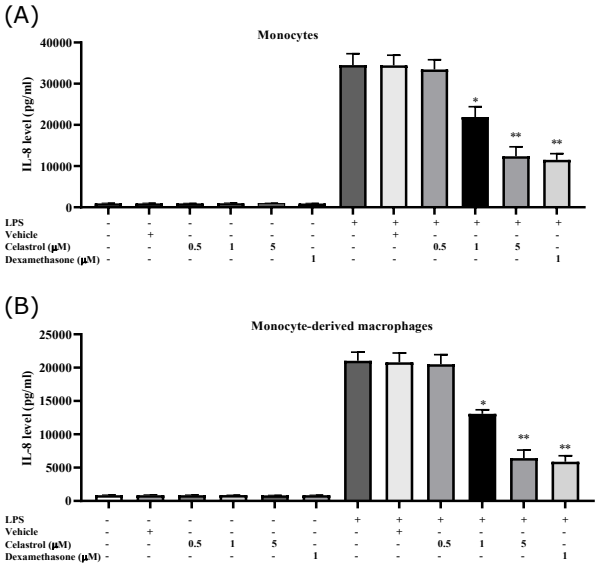

Fig. 3 Effects of celastrol on IL-8 levels in supernatants from LPS-activated monocytes (A) and monocyte-derived macrophages (B). Cells were treated with 0.5–5 μM of celastrol for 24 h and then activated by 10 ng/ml LPS for 12 h. DMSO was used as the vehicle control, and 1 μM dexamethasone was used as the positive control. Data are reported as mean ± SD. * $p < 0.05$; ** $p < 0.01$ vs. LPS activation ($n = 4$).

Effects of celastrol on IL-8 release by LPS-activated monocytes and monocyte-derived macrophages

To determine whether celastrol inhibited IL-8 production in monocytes and monocyte-derived macrophages after the LPS challenge, cells were treated with various concentrations of celastrol for 24 h and then activated with LPS. In monocytes, LPS treatment for 12 h resulted in maximum production of IL-8 of up to 35,000 pg/ml cultured medium; whereas the untreated group and the celastrol or vehicle alone groups without LPS activation produced <1,000 pg/ml. Thus, there was minimal constitutive production of IL-8 by human monocytes, and this was unaffected by celastrol treatment. In contrast, celastrol at 1 and 5 μM significantly attenuated LPS-induced IL-8 release by human monocytes (Fig. 3A). The inhibition was dose-dependent with a half maximal inhibitory concentration (IC_{50}) of 3.13 ± 0.03 μM. Similarly, LPS-activated monocyte-derived macrophages released IL-8 at concentrations up to 20,000 pg/ml; whereas constitutive production of IL-8 by these cells was minimal and was not affected by pre-treatment with drugs. Celastrol at 1 and 5 μM significantly inhibited LPS-induced IL-8 release by monocyte-derived macrophages (Fig. 3B) with an IC_{50} of 3.18 ± 0.05 μM. Dexamethasone, a positive control, also strongly inhibited IL-8 production in LPS-treated cells.

Effects of celastrol on IL-8 mRNA expression by LPS-activated monocytes and monocyte-derived macrophages

IL-8 mRNA expression was measured to determine whether the inhibitory effects of celastrol on the production of IL-8 resulted from the modulation of IL-8 mRNA levels. IL-8 mRNA increased markedly in response to LPS treatment, while expression in untreated cells was low. In LPS-activated monocytes and monocyte-derived macrophages, celastrol at 1 and 5 μM significantly inhibited IL-8 mRNA expression in a dose-dependent manner (Fig. 4A, Fig. 4B). Dexamethasone, a positive control, also strongly inhibited IL-8 mRNA expression in LPS-treated cells. Neither celastrol nor dexamethasone affected the expression of the housekeeping gene β-actin. These results were consistent with the inhibitory effects of celastrol on IL-8 release by LPS-activated monocytes and monocyte-derived macrophages.

Effects of celastrol on NF-κB activation of LPS-activated monocytes and monocyte-derived macrophages

To define the molecular mechanism of celastrol-mediated inhibition of IL-8 release, we further char-

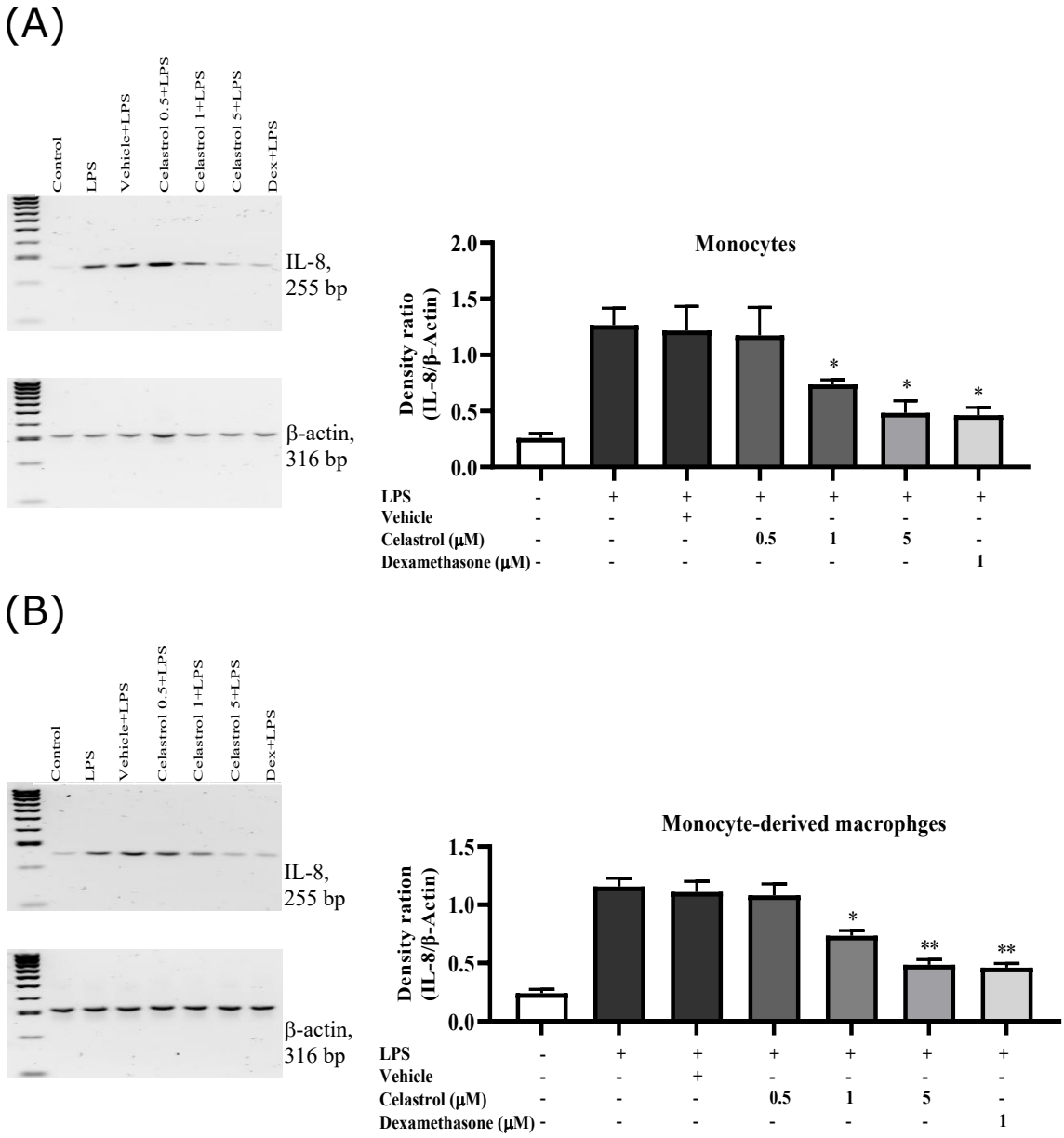

Fig. 4 IL-8 mRNA levels in LPS-treated monocytes (A) and LPS-treated monocyte-derived macrophages (B) were determined by RT-PCR. The expression of the housekeeping gene, β-actin, was the internal control. The density of the IL-8 mRNA band was normalized to β-actin. Data are reported as mean ± SD. * $p < 0.05$; ** $p < 0.01$ vs. LPS activation ($n = 4$).

acterized its effect on LPS-stimulated NF-κB activation. LPS stimulation for 45 min resulted in phosphorylation of IκB kinase (IKK), inhibition of kappa B-α (IκBα), and a marked increase in p-p65. Treatment with 1 μM or 5 μM celastrol or 1 μM gliotoxin significantly inhibited p-IKK, p-IκBα, and p-p65 subunit activation by LPS. While there was no significant change in total protein levels of IKK and p65, there was a dramatic reduction in the IκBα subunit by treatment with the NF-κB inhibitor gliotoxin. Celastrol at 1 μM or 5 μM, but not at 0.5 μM, also blocked the LPS-induced loss of the IκBα subunit in monocytes (Fig. 5A). Similar results were observed in LPS-activated monocyte-derived macrophages; protein levels of p-IKK, p-IκBα, and p-p65 were significantly increased by LPS activation, and that increase was inhibited by 1 μM and 5 μM celastrol (Fig. 5B). The dramatic loss of the cytoplasmic IκBα subunit was suppressed by 1 μM and 5 μM celastrol, but not by 0.5 μM celastrol. The vehicle control (0.2% DMSO) did not affect the celastrol-mediated inhibition

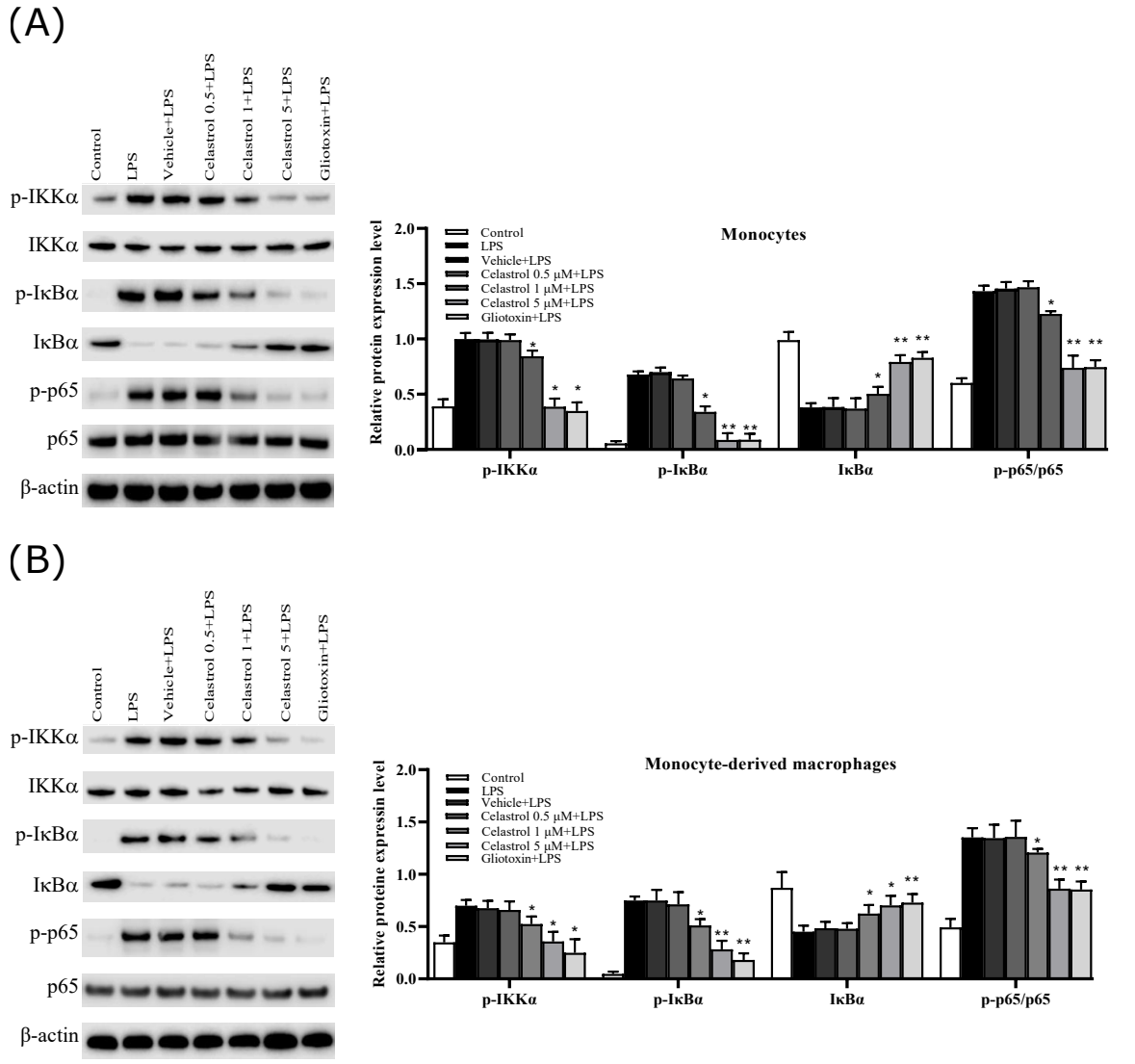

Fig. 5 Effect of celastrol on the LPS-induced NF-κB signalling pathway activation in monocytes (A) and monocyte-derived macrophages (B). Cells were pre-treated with 0.5, 1, 5 μM celastrol, DMSO (vehicle), and gliotoxin (NF-κB inhibitor, positive control) for 24 h; then, activated with 10 ng/ml LPS for 45 min. The protein levels for total and phosphorylated IKKα, IκBα, and p65 were determined by Western blot. β-actin was the endogenous control. Data are reported as mean ± SD. * $p < 0.05$; ** $p < 0.01$ vs. LPS activation (n =3).

of NF-κB activation, and no treatment affected the control β-actin protein.

DISCUSSION

The pentacyclic triterpene celastrol appears to have therapeutic potential in many inflammatory diseases. We examined the inhibitory action of celastrol on LPS-induced IL-8 production by human monocytes and monocyte-derived macrophages and the potential molecular signalling pathway involved in its action. We found that celastrol contributed to anti-inflammatory effects by inhibiting IL-8 release via suppression of the NF-κB signalling pathway.

While the constitutive level of IL-8 production by monocytes and monocyte-derived macrophages was unaffected by any drug treatment, celastrol at 1–5 μM significantly inhibited LPS-induced production of IL-8 by monocytes and monocyte-derived macrophages. The anti-inflammatory drug dexamethasone was the positive reference drug, and it also strongly inhibited IL-8 production in LPS-treated cells. Loss of IL-8 after celastrol treatment was associated with a decrease in IL-8 mRNA levels. These results are consistent with the studies of Shi et al [23] and Wei and Wang [24], who proposed that celastrol reduces IL-8 levels in LPS-induced acute respiratory distress syndrome.

LPS activates the transcriptional factor NF-κB, a critical regulator of inflammatory cytokines such as IL-8 in many cell types, including monocytes and macrophages [20, 25, 26]. In resting cells, NF-κB proteins are predominantly cytoplasmic, associating with the inhibitory IκB family members such as IκBα [27]. Activation of NF-κB classically depends on IκBα degradation, which requires the prior phosphorylation of IκB by IκB kinases (IKKs) [28]. IKK activity resides in a large protein complex comprising two catalytic subunits, IKKα and IKKβ. Activators of the IKK complex include mitogen-activated protein kinases, which represent a convergence point for numerous stimuli, including ligands for TLRs such as LPS.

The anti-inflammatory property of celastrol by suppression of NF-κB signalling has been demonstrated in both *in vivo* and *in vitro* models. Zang et al [29] found that celastrol attenuated renal injury in diabetic rats via NF-κB signalling, while Luo et al [30] reported that celastrol suppressed macrophage polarization against inflammation in diet-induced obese mice by regulating NF-κB pathways. Additionally, Zang et al [31] demonstrated amelioration of inflammation in human retinal epithelial cells by suppression of NF-κB signalling; and Jung et al [32] reported that celastrol inhibited the production of cytokines through NF-κB in LPS-stimulated microglial cells. Moreover, Veerappan et al [33] found that celastrol inhibited the phosphorylation of IκB and p65 subunits by inhibiting IKK activity in human neuroblastoma cells. Here, we demonstrated that celastrol suppressed the NF-κB signalling activation by inhibiting phosphorylated IKK, IκBα, and p65 while preventing the IκBα degradation resulted from LPS-induced IL-8 production by monocytes and monocyte-derived macrophages.

CONCLUSION

The present studies provided evidence that celastrol significantly inhibited LPS-induced IL-8 production by human monocytes and monocyte-derived macrophages. The inhibitory effects on the production of IL-8 were related to the modulation of IL-8 mRNA levels. A possible mechanism for this anti-inflammatory action was through inhibition of the NF-κB signalling pathway by celastrol. Thus, celastrol is a potential anti-inflammatory agent for the treatment of a wide range of inflammatory conditions.

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