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16α, 17α-epoxypregnenolone-20-oxime inhibits NO and IL-6 production in LPS-treated RAW264.7 cells

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Abstract. It has previously been reported that 16α, 17α-epoxypregnenolone-20-oxime (EPREGO) exerts an inhibitory effect on nitric oxide (NO) production and inducible NO synthase (iNOS) expression in microglia. The present study aimed to investigate the effects of EPREGO on the lipopolysaccharide (LPS)-induced inflammatory response in RAW264.7 macrophage cells, and to determine the underlying molecular mechanisms using western blot analysis, enzyme-linked immunosorbent assays and fluorescence-activated cell sorting. The present study demonstrated that LPS-induced production of NO and interleukin (IL)-6, and the protein expression levels of iNOS, were reduced by EPREGO in a dose- and time-dependent manner, whereas, EPREGO did not affect tumor necrosis factor-α production. In addition, EPREGO suppressed LPS-induced cellular reactive oxygen species production and phagocytosis. Furthermore, EPREGO significantly inhibited the LPS-induced activation of mitogen-activated protein kinases and inhibitor of κB α degradation in LPS-stimulated RAW264.7 cells, thus resulting in modulation of the production of NO and IL-6. Taken together, these results suggest that EPREGO exhibits anti-inflammatory activity in macrophages, thus validating the hypothesis that EPREGO may be useful as a therapeutic agent for the treatment of macrophage-mediated inflammation.

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Abbreviations: LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; NADPH, nicotinamide adenine dinucleotide phosphate; EPREGO, 16α, 17α-epoxypregnenolone-20-oxime

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Key words: 16α, 17α-epoxypregnenolone-20-oxime, nitric oxide, IL-6, LPS, inflammation

Introduction

Macrophages, which are critical effector cells, contribute to the innate immune response against infection. Macrophages are considered the most efficient pathogen scavengers, and are the main source of proinflammatory mediators and cytokines, including nitric oxide (NO), interleukin (IL)-6 and tumor necrosis factor (TNF)-α. Proinflammatory mediators are essential for inducing inflammation at the site of infection and for fighting pathogenic infections (1,2). However, the excessive production of proinflammatory cytokines has severe consequences, including tissue damage and septic shock (3,4). Therefore, suppressing the synthesis or release of proinflammatory cytokines and mediators is a potential therapeutic strategy for the treatment of septic shock-like diseases associated with inappropriately amplified inflammation.

Several intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways, are activated in lipopolysaccharide (LPS)-induced macrophages, and regulate inflammatory actions and immune responses. In macrophages, the MAPK signaling pathway is one of the most extensively investigated intracellular signaling cascades involved in the LPS-induced inflammatory response (5-8). The MAPK pathway is comprised of at least three signaling components: Extracellular signal-regulated kinases 1/2 (ERK 1/2); c-Jun N-terminal kinase (JNK); and p38 MAPK, all of which have been demonstrated to induce the release of immune-related cytotoxic factors and proinflammatory cytokines (9-11). Furthermore, NF-κB is important for controlling innate and adaptive immunity, and for regulating the expression of various genes during the inflammatory response (12).

Activated NF-κB translocates into the nucleus of the cell and interacts with κB-binding sites in the promoter regions of target genes, in order to regulate the transcription of proinflammatory genes, including inducible nitric oxide synthase (iNOS), TNF-α and IL-6 (13,14).

LPS-stimulated NO production and iNOS expression have previously been reported to be inhibited by 16α, 17α-epoxypregnenolone-20-oxime (EPREGO) in BV2 microglial cells via downregulation of the JNK signaling pathway (15). These results indicated that EPREGO may be associated with neuroinflammation. EPREGO is an organic compound derived from 16-dehydropregnenolone-3-acetate (commonly known as 16-DPA); however, the mechanisms underlying the anti-inflammatory activity of EPREGO in macrophages remain unclear. The present study aimed to investigate the anti-inflammatory effects of EPREGO on LPS-stimulated macrophages.

Materials and methods

Chemicals. LPS (from *Escherichia coli* serotype 0111:B4) and inhibitors of p38 MAPK (SB203580), ERK (PD98059) and JNK (SP600125) were all purchased from Calbiochem (San Diego, CA, USA). EPREGO, a steroid oxime syntheses product derived from 16-dehydropregnenolone-3-acetate, was obtained as previously reported (15).

Cell culture. The RAW264.7 macrophages, immortalized mice macrophage cells (Shanghai BOGO Industrial Co., Ltd., Shanghai, China), was propagated in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and (100 U/ml penicillin-100 μg/ml streptomycin (HyClone, GE Healthcare Life Sciences). Exponentially growing RAW264.7 cells maintained in DMEM at 37˚C and 5.0% CO_2 were pretreated with 10 μg/ml EPREGO, followed by 1 μg/ml LPS for the indicated times (0, 1, 3, 6, 12 and 24 h).

Biochemical assay for the production of NO. NO production was assessed based on the accumulation of nitrite in the medium using a colorimetric reaction with Griess reagent. Culture supernatants were collected and mixed with an equal volume of Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 0.1% sulfanilamide and 2.5% H_3PO_4]. Absorbance was measured at 540 nm using a UV MAX kinetic microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Cell cytotoxicity analysis. RAW 264.7 cells were seeded into a 96-well dish ($1.6x10^6$ cells/ml) and exposed to 1, 5 or 10 μg/ml EPREGO without LPS for 24 h. A solution of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) was subsequently added to each well and the cells were incubated for 2 h at 37˚C in an atmosphere containing 5% CO_2. Subsequently, the supernatant was removed and formazan was solubilized with dimethyl sulfoxide. Absorbance was measured at 540 nm using a UV MAX kinetic microplate reader (Molecular Devices, LLC).

Western blotting. Protein lysates (30 μg) were extracted with protein lysis buffers (20 mM HEPES-OH, pH 7.0; 50 mM NaCl, 10% glycerol and 0.5% Triton X-100) and incubated with 0.5 μg/ml leupeptin; 0.7 μg/ml pepstatin A; 0.1 mM 4-(2-aminoethyl)-benzenesulfony fluoride and 2 μg/ml aprotinin for 30 min at 4˚C. The proteins were then denatured for 5 min and separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk for 30 min, at room temperature. They were then incubated with polyclonal rabbit anti-iNOS (cat. no. 06-573, EMD Millipore, Billerica, MA, USA), polyclonal rabbit anti-IκB-α (cat. no. sc-371), mouse monoclonal anti-p-JNK (cat. no. sc-6254), anti-JNK (cat. no. sc-7345), mouse monoclonal anti-p-P38 (cat. no. sc-7973), mouse monoclonal anti-P38 (cat. no. sc-7972), mouse monoclonal anti-p-ERK (cat. no. sc-7383), and mouse monoclonal anti-ERK (cat. no. sc-514302) (all obtained from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (cat. no. PLA0125, Sigma-Aldrich) primary antibodies (dilution, 1:5,000) at 4˚C overnight. The membranes were washed five times with Tris-buffered saline (TBS) containing Tween [10 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.2% Tween-20] and were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit (cat. no. SAB3700878, 1:10,000) or anti-mouse immunoglobulin G (cat. no. SAB3701105, 1:10,000) (Sigma-Aldrich) for 1 h at room temperature. Following the removal of excess antibodies by washing with TBS, specific binding was detected using a chemiluminescence detection system (GE Healthcare Life Sciences, Chalfont, UK) according to the manufacturer's protocol.

Cytokine assays. The concentration of TNF-α and IL-6 in the cell culture supernatant was measured using enzyme-linked immunosorbent assay (ELISA) kits for TNF-α and IL-6 (eBioscience, Inc., San Diego, CA, USA). RAW264.7 cells ($2.5x10^5$ cells) were plated into a 48-well cell culture plate and incubated with various concentrations of EPREGO (1, 5 and 10 μg/ml) and 1 μg/ml LPS for 24 h. The culture supernatant was collected and assayed according to the manufacturer's protocols, in order to determine the concentration of TNF-α and IL-6 that had been released from the cells.

Measurement of phagocytosis and ROS by flow cytometry. Macrophage phagocytosis was analyzed with flow cytometry, according to a previously reported method (16). Briefly, Alexa 488-conjugated *E. coli* (Ec-A) BioParticles (Invitrogen; Thermo Fisher Scientific, Inc.) were sonicated, added to the culture medium without serum at the final time of LPS treatment and incubated at 37˚C for 15 min. Following incubation, the cells were washed with phosphate-buffered saline (PBS) three times and were resuspended in 500 μl PBS. The internalized fluorescence was immediately determined from 10,000 cells using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). To determine ROS levels, RAW264.7 cells were incubated with 10 mM CM-H_2DCFDA (Invitrogen; Thermo Fisher Scientific, Inc.), a fluorescence-based ROS indicator, at 37˚C for 15 min at

the final time of various treatments, and the DCFDA fluorescence intensity from 10,000 cells was measured using FACScan (BD Biosciences). The results were analyzed using WinMDI (Version 2.9, BD Biosciences) software.

Statistical analysis. The data are presented as the mean ± standard error of the mean. Differences between experimental groups were analyzed by one-way analysis of variance and a Tukey test. GraphPad Prism software version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze results. P<0.01 was considered to indicate a statistically significant difference.

Results

EPREGO inhibits the production of proinflammatory cytokines in LPS-treated RAW264.7 cells. The chemical structure of EPREGO is presented in Fig. 1A. EPREGO is a steroid oxime that has previously been reported to exert an inhibitory effect on LPS-stimulated NO production in BV2 microglia cells (15). To examine the effects of EPREGO on macrophage cell viability, RAW264.7 cells were pretreated with various concentrations of EPREGO for 24 h, then stimulated with LPS for 12 h. Cell viability was detected using an MTT assay. As demonstrated in Fig. 1B, cell viability was not affected up to a concentration of 10 μg/ml, whereas, a higher concentration of EPREGO (20 μg/ml) significantly reduced the viability of RAW264.7 cells compared with untreated cells (P=0.008). Therefore, in subsequent studies 10 μg/ml was used as the highest treatment concentration. To evaluate whether EPREGO has anti-inflammatory properties, the NO, IL-6 and TNF-α levels in RAW264.7 cell culture supernatants were examined by ELISA following stimulation with LPS. Compared with LPS-stimulated cells, treatment with EPREGO dose- and time-dependently decreased the production of NO (P=0.009, P=0.0003 and P=0.0006 for 6, 12 and 24 h, respectively) and IL-6 (P=0.009, P=0.0003 and P=0.0006 for 6, 12 and 24 h, respectively) in LPS-treated RAW264.7 cells (Fig. 2), but did not markedly alter TNF-α secretion (Fig. 3). Furthermore, the results of western blotting experiments demonstrated that, compared with LPS stimulation, EPREGO inhibited iNOS (an enzyme involved in NO production) protein expression in a dose- and time-dependent manner (Fig. 4A and B), resulting in decreased NO production.

EPREGO decreases cellular ROS levels and macrophage phagocytosis in LPS-stimulated RAW264.7 cells. To investigate whether EPREGO affects the ROS levels and phagocytic capacity of LPS-stimulated macrophages, RAW264.7 cells were pretreated with EPREGO (10 μg/ml) for 30 min, followed by treatment with LPS (1 μg/ml) for 24 h. Cellular ROS levels and phagocytosis were examined using flow cytometry. LPS increased cellular ROS levels, which were significantly decreased by treatment with EPREGO, almost to basal levels compared with LPS-treated cells (Fig. 4C, P=0.00011). Similarly, treatment with EPREGO significantly decreased phagocytosis compared with LPS-stimulated RAW264.7 cells (Fig. 4D, P=0.008). Together these results (Figs. 2-4) demonstrate that EPREGO may exert anti-inflammatory effects in RAW264.7 cells.

EPREGO exhibits anti-inflammatory effects by inhibiting MAPK signaling pathways. The MAPK and NF-κB signaling pathways are crucial mediators of proinflammatory cytokine production in macrophages (9-12). To determine the mechanism underlying the anti-inflammatory activities of EPREGO in RAW264.7 cells, the MAPK pathways were investigated by detecting the phosphorylation levels of ERK, JNK and p38. RAW264.7 cells were pretreated with EPREGO (10 μg/ml) for 30 min, followed by treatment with LPS (1 μg/ml) for the indicated times, and MAPK phosphorylation was subsequently examined using western blotting. The results demonstrated that treatment with LPS upregulated ERK, p38 and JNK phosphorylation compared with in the untreated cells, whereas phosphorylation was markedly downregulated by EPREGO treatment compared with LPS-treated cells (Fig. 5A-C). Furthermore, the inhibitory effect of EPREGO on the NF-κB signaling pathway was investigated by examining IκBα degradation. IκBα degradation in RAW264.7 cells was markedly inhibited by EPREGO treatment compared with LPS-stimulated cells (Fig. 5D). To determine whether the MAPK signaling pathways were associated with the anti-inflammatory effects of EPREGO on LPS-stimulated macrophage inflammation, the effects of pharmaceutical MAPK inhibitors were assessed. The results demonstrated that treatment with SB203580 (p38 inhibitor), PD98059 (ERK inhibitor) and SP600125 (JNK inhibitor), as well as EPREGO, significantly decreased NO (P=0.008, P=0.0003, P=0.004 and P=0.0002 for SB203580, PD98059, SP600125 and EPREGO, respectively) and IL-6 (P=0.0003, P=0.0007, P=0.0002 and P=0.0003 for SB203580, PD98059, SP600125 and EPREGO, respectively) production in RAW264.7 cells compared with LPS treatment (Fig. 6A and B), whereas TNF-α secretion was not markedly altered (Fig. 6C). These results indicate that MAPK signaling pathways are associated with the inhibitory effects of EPREGO on NO and IL-6 production in LPS-treated RAW264.7 cells.

Discussion

Inflammation is the host response to infection and injury; however, if uncontrolled, inflammatory mediators become involved in the pathogenesis of various inflammatory disorders (17). Proinflammatory cytokines that are typically released by macrophages are critical for initiating and sustaining the inflammatory response.

Following processing, 16-dehydropregnenolone-3-acetate, a major intermediary for hormone- and steroid-associated drugs (18,19), exhibits anti-inflammatory, anti-toxin, anti-shock and anti-allergenic effects. EPREGO, which is derived from 16-dehydropregnenolone-3-acetate, was previously reported to inhibit NO production in LPS-stimulated BV2 microglia cells (15); however, to the best of our knowledge, its potential anti-inflammatory properties have not been investigated in macrophages. The present study investigated the effects of EPREGO on proinflammatory cytokine production in LPS-stimulated RAW264.7 macrophages. Treatment with EPREGO significantly decreased LPS-stimulated NO and IL-6 production (Fig. 2), and iNOS protein expression, but did not alter TNF-α production (Fig. 3) in RAW264.7 cells. Furthermore, EPREGO reduced

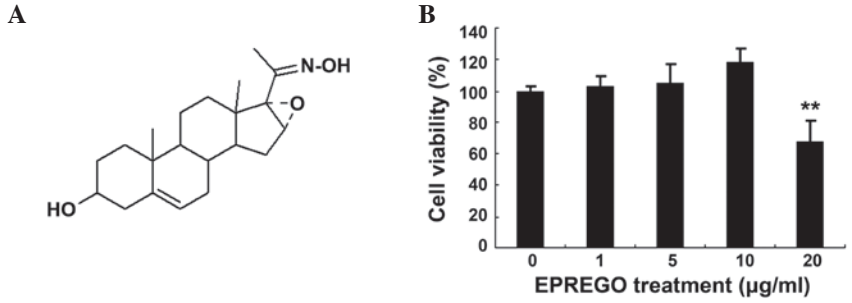

Figure 1. EPREGO and its effect on RAW264.7 cell viability. (A) Chemical structure of EPREGO. (B) Cells were treated with the indicated concentrations of EPREGO and incubated for 24 h. Cell viability was measured using an MTT assay. Data are presented as the mean ± standard error of the mean. EPREGO, 16α, 17α-epoxypregnenolone-20-oxime. **P<0.01 vs. 0 μg/ml EPREGO-treated cells.

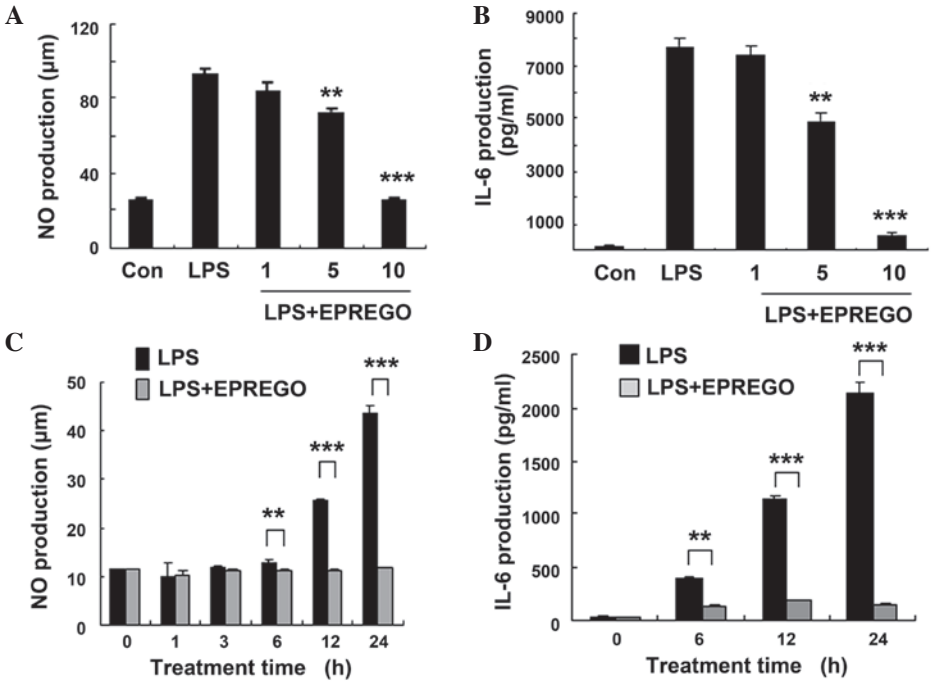

Figure 2. Effects of EPREGO on NO and IL-6 production. Cells were pretreated with the indicated concentrations of EPREGO for 30 min prior to LPS (1 μg/ml) stimulation and were incubated for 24 h. The levels of (A) NO and (B) IL-6 production were measured in the culture media using Griess reagent and enzyme-linked immunosorbent assay kits. In addition, the cells were pretreated with 10 μg/ml EPREGO for 30 min, followed by treatment with LPS (1 μg/ml) for the indicated durations, and the secretion of (C) NO and (D) IL-6 were detected. Data are presented as the mean ± standard error of the mean of three different samples. **P<0.01 and ***P<0.001 vs. LPS-stimulated cells. NO, nitric oxide; LPS, lipopolysaccharide; EPREGO, 16α, 17α-epoxypregnenolone-20-oxime; IL-6, interleukin-6.

the LPS-induced cellular ROS levels and phagocytosis of RAW264.7 cells (Fig. 4). These observations are consistent with previous reports (15), indicating that EPREGO exerts anti-inflammatory activity in RAW264.7 cells.

Mechanistic analysis within the present study demonstrated that EPREGO may significantly downregulate LPS-stimulated phosphorylation of ERK, JNK and p38 (Fig. 5A-C), thus indicating that the inhibitory activity of EPREGO on the production of NO and IL-6 in RAW264.7 cells is associated with the MAPK signaling pathway. Upon LPS recognition of complex proteins, including LPS binding protein, cluster of differentiation 14, lymphocyte antigen 96 and Toll-like receptor (TLR) 4, a series of TLR-mediated signal pathways activate downstream IκB kinase (IKK) and MAPK pathways (20). The NF-κB and MAPK pathways are the major intracellular signaling pathways activated by LPS binding to TLR4 on the cell membrane (5,21). MAPKs are a family of serine/threonine protein kinases responsible for most cellular responses to cytokines, which are crucial for regulating the production of inflammatory mediators (22-25). As previously reported, EPREGO inhibits NO production and iNOS expression by selectively downregulating JNK phosphorylation (15). The present study demonstrated that treatment with EPREGO inhibits the phosphorylation of JNK, ERK and p38 in RAW264.7 cells (Fig. 5A-C). The differential effects of EPREGO on MAPK (ERK, JNK and p38) signaling pathways between microglia and macrophages may be due to the different characteristics of the two cell types, and the mechanism for this regulation should be investigated further. In addition, treatment of RAW264.7 cells with pharmaceutical inhibitors for ERK (PD98059), JNK (SP600125) and p38 (SB203580) inhibited the production of

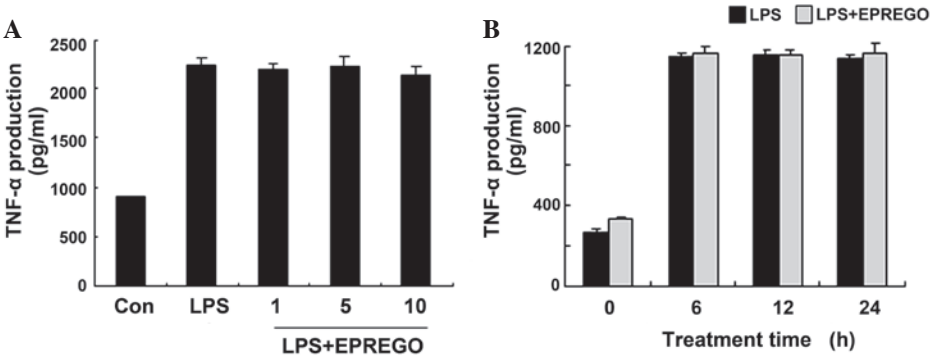

Figure 3. Effects of EPREGO on LPS-induced TNF-α production. (A) Cells were pretreated with the indicated concentrations of EPREGO for 30 min prior to LPS (1 μg/ml) stimulation and were incubated for 24 h. (B) In addition, the cells were pretreated with 10 μg/ml EPREGO for 30 min, followed by treatment with LPS (1 μg/ml) for the indicated durations. The levels of TNF-α production were measured in the culture media by enzyme-linked immunosorbent assay. Data are presented as the mean ± standard error of the mean for three different samples. TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; EPREGO, 16α, 17α-epoxypregnenolone-20-oxime

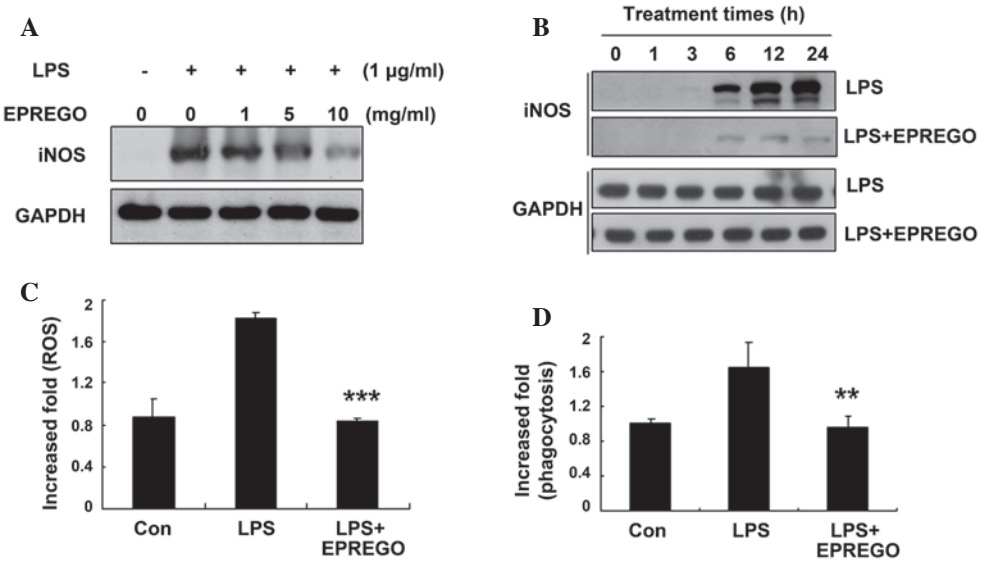

Figure 4. EPREGO inhibits iNOS protein expression, ROS production and phagocytosis. Cells were treated with (A) the indicated concentrations (B) for the indicated durations, and the protein expression levels of iNOS were determined by western blotting. To examine the effects of EPREGO on macrophage ROS and phagocytosis, RAW264.7 cells were pretreated with EPREGO (10 μg/ml) for 30 min and were then stimulated with LPS (1 μg/ml) for 24 h. The levels of (C) ROS and (D) phagocytosis were detected using flow cytometry. The fold increase of ROS and phagocytosis compared to untreated cells was calculated. Data are presented as the mean ± standard error of the mean. The experiments were conducted on three different samples. $^{**}P<0.01$ and $^{***}P<0.001$ vs. LPS-stimulated cells. LPS, lipopolysaccharide; EPREGO, 16α, 17α-epoxypregnenolone-20-oxime; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

NO and IL-6 (Fig. 6A and B). These results suggested that EPREGO may alter the MAPK signaling cascade, resulting in decreased NO and IL-6 production.

NF-κB is a major transcription factor that regulates the expression of genes responsible for the innate and adaptive immune responses. The inappropriate regulation of NF-κB has been associated with cancer, and inflammatory and autoimmune diseases. NF-κB has been demonstrated to be important for the inflammatory response and the expression of inflammatory cytokines, including NO, TNF-α and IL-6. NF-κB activation is induced by the dissociation of IκBα/β, which are phosphorylated by IKK. The level of IκBα and IκBβ degradation is commonly used as an indicator of NF-κB activation (26). In the present study, EPREGO was demonstrated to inhibit IκBα degradation in LPS-stimulated RAW264.7 cells (Fig. 4D), which indicated that EPREGO potentially suppresses the NF-κB signaling pathway, thus, reducing inflammatory response-mediated NO and IL-6 production. These results are consistent with previous reports demonstrating that the NF-κB signaling pathway is essential for LPS-stimulated cytokine production (27-30) in macrophages.

In the present study, although EPREGO inhibited IκBα degradation and the phosphorylation of MAPKs (Fig. 5), it had no effect on TNF-α production (Fig. 3), thus suggesting that LPS-induced TNF-α secretion is influenced by other signaling pathways. Indeed, it was previously reported that the Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) signaling pathway is involved in proinflammatory cytokine and ROS production (31-33); therefore,

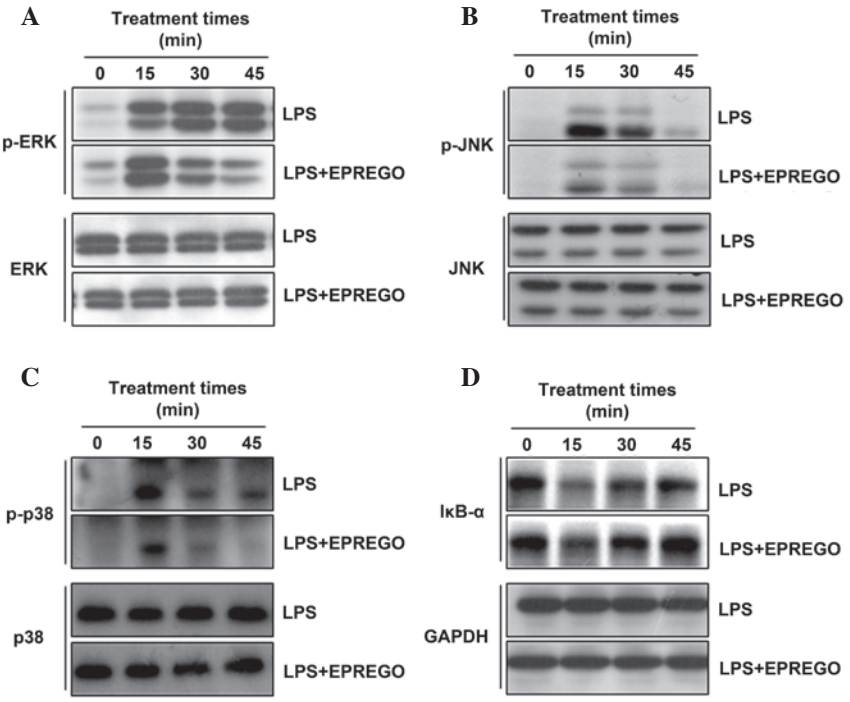

Figure 5. Effects of EPREGO on LPS-stimulated mitogen-activated protein kinase phosphorylation and IκBα degradation. RAW264.7 cells were pretreated with EPREGO (10 μg/ml) for 30 min, followed by treatment with LPS (1 μg/ml) for the indicated durations. The phosphorylation levels of (A) ERK, (B) JNK and (C) p38, and (D) IκBα degradation were detected using western blotting. The experiments were conducted on three different samples. LPS, lipopolysaccharide; p, phosphorylated; ERK, extracellular signal-regulated kinase; EPREGO, 16α, 17α-epoxypregnenolone-20-oxime; IκBα, inhibitor of κB α; JNK, c-Jun N-terminal kinase; IκBα, inhibitor of κB α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

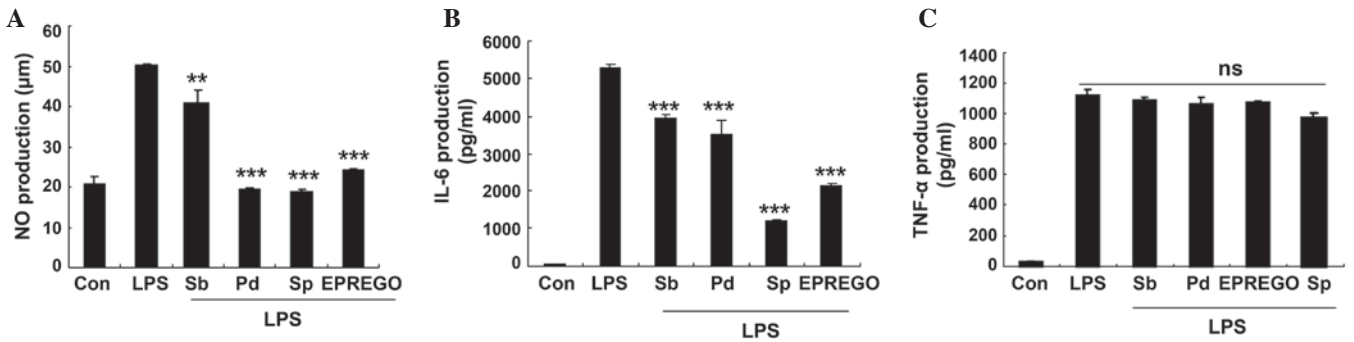

Figure 6. Mitogen-acitivated protein kinase signaling pathway inhibitors reduced LPS-stimulated NO and IL-6 secretion. RAW264.7 cells were pretreated with SB203580 (p38 inhibitor), PD98059 (extracellular signal-regulated kinase inhibitor) and SP600125 (c-Jun N-terminal kinase inhibitor) for 30 min followed by LPS (1 μg/ml) for 24 h. The levels of (A) NO, (B) IL-6 and (C) TNF-α were measured using Griess reagents and enzyme-linked immunosorbent assays. Data are presented as the mean ± standard error of the mean of three different samples. **P<0.01 and ***P<0.001 vs. LPS-stimulated cells. NO, nitric oxide; LPS, lipopolysaccharide; Sb, SB203580; Pd, PD98059; Sp, SP600125; EPREGO, 16α, 17α-epoxypregnenolone-20-oxime; IL-6, interleukin-6; TNF-α; tumor necrosis factor-α; ns, not significant.

it is hypothesized that TNF-α production may be regulated by the JAK/STAT signaling pathway, and an analysis of the inhibitory effect of EPREGO on LPS-stimulated JAK/STAT signaling is currently being investigated.

In conclusion, the results of the present study indicated that EPREGO attenuates the production of NO and IL-6 in LPS-stimulated RAW264.7 cells, and decreases cellular ROS levels and phagocytosis by inhibiting the MAPKs and NF-κB signaling pathways. The results suggest that EPREGO exhibits an anti-inflammatory role in macrophage cells, and may serve as a therapeutic agent for macrophage-mediated inflammation.

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