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Ethyl rosmarinate inhibits lipopolysaccharide-induced nitric oxide and prostaglandin E_2 production in alveolar macrophages

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ABSTRACT

In this study, a series of rosmarinic acid and analogs were investigated for their anti-inflammatory potential against LPS-induced alveolar macrophages (MH-S). Our results showed that, among the test compounds, ethyl rosmarinate (**3**) exhibited the most potent inhibitory effect on NO production in LPS-induced MH-S cells, with low cytotoxicity. Compound **3** exhibited remarkable inhibition of the production of PGE_2 in LPS-induced MH-S cells. The inhibitory potency of compound **3** against LPS-induced NO and PGE_2 release was approximately two-fold higher than that of dexamethasone. Compound **3** significantly decreased the mRNA and protein expression of iNOS and COX-2 and suppressed p65 expression in the nucleus in LPS-induced MH-S cells. These results suggested that compound **3** inhibited NO and PGE_2 production, at least in part, through the down-regulation of NF-κB activation. Analysis of structure-activity relationship revealed that the free carboxylic group did not contribute to inhibitory activity and that the alkyl group of the corresponding alkyl ester analogs produced a strong inhibitory effect. We concluded that compound **3**, a structurally modified rosmarinic acid, possessed potent inhibitory activity against lung inflammation, which strongly supported the development of this compound as a novel therapeutic agent for the treatment of macrophage-mediated lung inflammatory diseases, such as COPD.

1. Introduction

Lung inflammation is fundamental to the etiology and persistence of respiratory disease conditions, including asthma and chronic obstructive pulmonary disease (COPD). In particular, COPD is a major health problem that results in morbidity and mortality worldwide (Barnes, 2015). A recent systemic review and meta-analysis suggested a high and growing prevalence of COPD, both globally and regionally (Adeloye et al., 2015). Among the various cell types, alveolar macrophages and neutrophils are thought to be responsible for COPD-related inflammation (Barnes, 2004; Meijer et al., 2013). However, there is increasing evidence to show that alveolar macrophages play a key role in the pathogenesis of COPD (Barnes, 2004). COPD is a disease of the small airways and lung parenchyma, of which inflammation and tissue destruction are the pathological hallmarks (Di Stefano et al., 2004). Alveolar macrophages are the predominant cell type in the lung (Shapiro, 1999; Pons et al., 2005), and a marked increase (5–10 fold) in the numbers of macrophages both in airways and in lung parenchyma was evident in patients with COPD (Keatings et al., 1996; Pesci et al., 1998). Furthermore, the number of parenchymal alveolar macrophages, but not neutrophils, was found to be directly proportional to the severity of the disease (Finkelstein et al., 1995; Di Stefano et al., 1998). Moreover, the pathological role of alveolar macrophages has been demonstrated, as the depletion of lung macrophages conferred protection against the development of emphysema in an experimental model of COPD (Beckett et al., 2013). Upon activation by harmful stimuli (predominantly cigarette smoke and bacterial infections) alveolar macrophages release inflammatory mediators including nitric oxide (NO), prostaglandin E_2 (PGE_2) and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Barnes, 2016). The uncontrolled production or excessive accumulation of these mediators can destroy lung tissue, which leads to respiratory failure and dysfunction. As a vital organ for gaseous exchange, chronic or excessive inflammation of the lung can be life threatening. Currently, bronchodilators and inhaled corticosteroids are prescribed as the first-line therapies for COPD. However, owing to the limited improvements in

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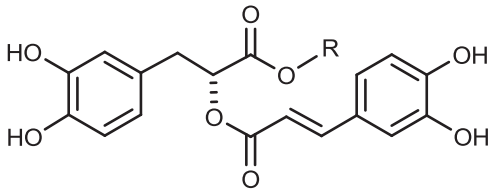
symptom control and survival rate (Callahan et al., 1991; Jiang and Zhu, 2016), this approach has not provided a satisfactory solution. More importantly, a certain proportion of patients with COPD are reported to be resistant to corticosteroids and experience a deterioration of their symptoms (Marwick et al., 2007; Malhotra et al., 2011; Jiang and Zhu, 2016). When used at high doses or for a prolonged time, corticosteroids can lead to problematic side effects, such as osteoporosis, aseptic joint necrosis, adrenal insufficiency, gastrointestinal, hepatic, and ophthalmologic effects, hyperlipidemia, growth suppression, and possible congenital malformations (Buchman, 2001). A significant increase in the risk of pneumonia with the long-term use of inhaled corticosteroids has also been reported in patients with COPD (Sonal and Loke, 2010). Therefore, the discovery and development of new agents for the effective treatment of COPD are an urgent necessity.

Rosmarinic acid (**1**), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, is commonly found in several medicinal plant species, including *Rosmarinus officialis* (Kim et al., 2015) and *Hyptis suaveolens* (Prawatsri et al., 2013). Interest in this compound has increased considerably in the last decade owing to its diverse pharmacological activities, including anti-oxidant (Kelm et al., 2000; Al-Musayeib et al., 2011), anti-cancer (Moore et al., 2016), anti-angiogenic (Cao et al., 2016), anti-viral (Arabzadeh et al., 2013), and anti-microbial properties (Abedini et al., 2013). Rosmarinic acid also exerts anti-inflammatory effects, as demonstrated by its ability to inhibit NO and PGE_2 release in macrophage-mediated inflammation by using RAW 264.7 murine macrophages of peritoneal origin as a model (Huang et al., 2009). In addition, the anti-allergic activity of rosmarinic acid was also been shown in an experimental model of allergic asthma (Costa et al., 2012; Liang et al., 2016). These results indicated the immunomodulatory properties of rosmarinic acid and suggested the possible use of this compound for the treatment of airway inflammation. Nevertheless, marked differences between the inflammatory cells involved in the pathology of asthma and COPD have been described (Ichinose, 2009; Moldoveanu et al., 2009). Moreover, there are functional and phenotypic differences among macrophages from different tissue sites (Guth et al., 2009; Karagianni et al., 2013; Hussell and Bell, 2014). As such, an appropriate therapeutic target for certain airway inflammatory diseases would be of significant concern. As alveolar macrophages and the release of inflammatory mediators play a pivotal role in the pathogenesis of COPD, and owing to the absence of scientific evidence addressing the effects of rosmarinic acid on alveolar macrophages, this study therefore investigated the anti-inflammatory potential of rosmarinic acid in LPS-induced mouse alveolar macrophage MH-S cell lines. We also designed and prepared analogs of this compound and examined their inhibitory activities. The underlying mechanism responsible for the inhibitory effect was also explored.

2. Materials and methods

2.1. Rosmarinic acid (1) and analogs 2–11

Rosmarinic acid (**1**) and methyl rosmarinate (**2**) (Fig. 1) are natural compounds isolated from the weeds *Hyptis sauveolens* by our group (Prawatsri et al., 2013). Structural modifications of rosmarinic acid to the corresponding ester analogs, compounds **3–11,** (Fig. 1) were achieved by conventional esterification of rosmarinic acid to the corresponding esters using appropriate alcohols, with concentrated sulfuric acid as a catalyst at room temperature, except for the benzyl ester **10** that was synthesized by coupling reaction between rosmarinic acid (**1**) and benzyl alcohol using EDCI [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide] and DMAP (4-dimethylaminopyridine) in triethylamine and dichloromethane. The products were purified by column chromatography (Merck silica gel 60, particle size < 0.063 mm) and the structures of the synthesized analogs were confirmed by NMR spectroscopic data (recorded on a Bruker AVANCE 400 FT NMR spectrometer, operating at 400 MHz), and ESI mass spectral data (measured



1, R = H (Rosmarinic acid)
2, R = CH_3 (Methyl rosmarinate)
3, R = CH_2CH_3 (Ethyl rosmarinate)
4, R = $CH_2CH_2CH_3$
5, R = $CH(CH_3)_2$
6, R = $CH_2CH_2CH_2CH_3$
7, R = $CH_2CH(CH_3)_2$
8, R = $CH_2CH_2CH(CH_3)_2$
9, R = $CH(CH_2CH_3)_2$
10, R = $CH_2C_6H_5$
11, R = CH_2CH_2OH

Fig. 1. Chemical structures of the test rosmarinic acid and analogs.

with a Finnigan LC-Q mass spectrometer) (see Supplementary data). The presence of the alkoxyl groups from the starting alcohols was evident from the NMR spectra of the esters. The purity of the compounds was approximately 95%. These compounds were dissolved with dimethyl sulfoxide (DMSO; ≥ 99.5%, Sigma, France) and further diluted in culture medium before bioassay.

2.2. Cell culture and conditions

Murine alveolar macrophage cell line MH-S was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute medium-1640 (RPMI-1640; HyClone, Utah, USA) supplemented with 10% fetal bovine serum (Gibco, South America), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; HyClone, Utah, USA), 2 mM L-glutamine (Gibco, Brazil) and 100 U/ml and penicillin and 100 µg/ml streptomycin (Gibco, USA) in a 5% CO_2 atmosphere at 37 °C.

2.3. Cell viability assay

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Mosmann, 1983). MH-S cells (1×10^5 cells/well) were plated in a 96-well plate (Nunc™, Roskilde, Denmark) and incubated for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were then exposed to test compounds (final concentration 25 µM) for 24 h. Cells without the test compounds and cells treated with dimethyl sulfoxide (DMSO) served as untreated and vehicle controls, respectively. After the incubation, culture supernatant was discarded and 20 µl MTT solution (5 mg/ml; Sigma, St. Louis, MO, USA) was added. Cells were incubated for another 3 h and the supernatant was removed, subsequently 100 µl of DMSO was added to solubilize the formazan. Absorbance of solubilized formazan solution was measured at 540 nm using a microplate reader (Rayto RT-2100C microplate reader, China). Percentage of viable viability was calculated according to the equation: (absorbance of treated cells/ absorbance of untreated cells) × 100.

2.4. Nitrite and PGE_2 measurements

MH-S cells (1×10^5 cells/well) were plated in a 96-well plate (Nunc™) and incubated for 1 h at 37 °C in an 5% CO_2 atmosphere.

Subsequently, the cells were treated with 1 μg/ml lipopolysaccharide (LPS; Sigma, St. Louis, MO, USA) in the presence or absence of the test compounds and incubated for 24 h (for NO) and 48 h (for PGE_2). The culture supernatant was collected and the nitrite (NO^{2-}), a stable product of NO was determined using the Griess reagent system (Promega Corporation, USA). Briefly, 50 μl of culture supernatant was reacted with 50 μl of 1% sulfanilamide in 5% phosphoric acid for 10 min at room temperature in the dark. Then, 50 μl of 0.1% naphthylethylenediamine dihydrochloride was added. After 10 min incubation at room temperature, the optical density was measured at 540 nm using a microplate reader (BioTek Synergy HT, USA) and nitrite concentration calculated against a sodium nitrite standard curve. The levels of PGE_2 in culture medium were quantified by enzyme-immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

MH-S cells (2×10^6 cells/well) were plated in 6-well plates (Nunc™) and incubated at 37 °C in 5% CO_2 for 1 h. Cells were stimulated with LPS (1 μg/ml; Sigma) in the presence or absence of compound **3** (2.5, 5, 10 and 25 μM). After 24 h-incubation, total cellular RNA was extracted using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentrations of extracted RNA were determined by Nanodrop spectrophotometer (NanoDrop Technologies, USA). Total RNA (2 μg) was reverse-transcribed to cDNA using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA) following the manufacturer's instructions. The resulting cDNA was used as template for PCR analysis. The primer sequences of target genes were: iNOS forward 5′-CTC AGC CCA ACA ATA CAA G-3′, reverse 5′-CTA CAG TTC CGA GCG TCA-3′, COX-2 forward 5′-AAG CCT TCT CCA ACC TCT-3′, reverse 5′-ACA CTC TGT TGT GCT CCC- 3′ and β-actin forward 5′-TGT TAC CAA CTG GGA CGA CA-3′, reverse 5′-AAG GAA GGC TGG AAA AGA GC-3′ (Gao et al., 2014). Amplification conditions were 25 cycles at 95 °C for 10 s, 61.7 °C for 40 s and 72 °C for 10 s (for iNOS and β-actin) and 95 °C for 3 s, 56.4 °C for 40 s and 72 °C for 10 s (for COX-2). The PCR products were separated by electrophoresis on 1% agarose gel for 35 min. Gels were then stained with 1 mg/ml ethidium bromide and visualized by UV illumination. The intensity of each band was determined by ImageJ software and the relative expression level of iNOS or COX-2 gene was calculated using β-actin gene as a reference housekeeping gene.

2.6. Western blotting analysis

MH-S cells (2×10^6 cells/well) were plated in 6-well plates (Nunc™) and incubated at 37 °C in 5% CO_2 for 1 h. Cells were stimulated with LPS (1 μg/ml; Sigma) in the presence or absence of compound **3** (2.5, 5, 10 and 25 μM) for 30 min (for NFκB p65), 24 h (for iNOS) and 48 h (for COX-2). Cells were scraped into 1.5 ml-microcentrifuge tubes, washed with cold phosphate-buffered saline (PBS) and pellets. The cell pellets were then lysed using RIPA lysis buffer (Amresco, OH, USA) containing Halt™ protease and phosphatase inhibitor cocktails (Pierce Biotechnology, IL, USA). Nuclear and cytosolic proteins were extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology) and a Halt™ protease and phosphatase inhibitor cocktails (Pierce Biotechnology). Protein concentration was determined using a Bradford Protein assay kit (Bio-Rad). The extracted proteins (25 μg) subjected to electrophoresis using 10% Novex™ NuPAGE™ Bis-Tris protein gels (Invitrogen, USA). Separated proteins were transferred to nitrocellulose membranes (Bio-Rad, Germany) and non-specific bindings were blocked with 5% skim milk in TTBS (20 mM Tris, 150 mM NaCl, 0.1% Tween20) for 1 h at room temperature. The membranes were then incubated overnight with primary antibodies specific to iNOS (sc-7271, Santa Cruz Biotechnology, USA), COX-2 (sc-1745, Santa Cruz Biotechnology), NF-κB p65 (sc-8008, Santa Cruz Biotechnology) and β-actin (ab-170325, Abcam). The membranes were washed with TTBS twice and subsequently incubated with peroxidase-conjugated AffiniPure goat anti-mouse IgG (115-035-003, Jackson ImmunoResearch Laboratories, USA) or horseradish peroxidase-conjugated donkey anti-goat IgG (sc-2020, Santa Cruz Biotechnology), as appropriate. After 1 h incubation at room temperature, the immunoreactive bands were detected by Clarity™ Western ECL Blotting Substrates (Bio-Rad, USA) according to the manufacturer's instructions. Chemiluminescent signals were visualized using ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences, UK). The relative intensities of the protein bands were measured by ImageJ software and then normalized with β-actin.

2.7. Statistical analysis

Experimental values were presented as mean ± S.E.M. of at least two independent experiments. Statistical analyses were carried out Student's *t*-test using SPSS version 23.0 statistical program (Chicago, IL, USA). The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effect of rosmarinic acid (1) and analogs 2–11 on cell viability

The effect of rosmarinic acid (**1**) and the analogs **2–11** on MH-S macrophage cell viability was examined by using an MTT assay. As shown in Fig. 2, the viabilities of cells exposed to the test compounds at 25 μM showed individual variability. A clear decrease in viability was observed after the cells were treated with compounds **4**, **5**, **6**, **7**, **8**, **9** and **10**, compared with the untreated control. However, no changes in viability were seen after exposure to compounds **1**, **2**, **3** and **11**, which suggested that these compounds were not cytotoxic to MH-S macrophage cells. Furthermore, no differences in cell viability were found between the vehicle and untreated control.

3.2. Effect of rosmarinic acid (1) and analogs 2–11 on LPS-induced NO production in MH-S macrophages

Initially, 25 μM rosmarinic acid (**1**) and analogs **2–11** were investigated for their inhibitory activities against LPS-induced NO production in MH-S macrophages and the results are shown in Fig. 3A. The treatment with compounds **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9** and **10** markedly reduced the LPS-stimulated production of NO compared with the production in the LPS-treated group. Compound **11** exhibited a mild inhibitory effect, whereas compound **1** had no inhibitory effect. Through the combination of inhibitory activity and cytotoxicity, compounds **2** and **3** were selected for further assessment of their dose-dependent

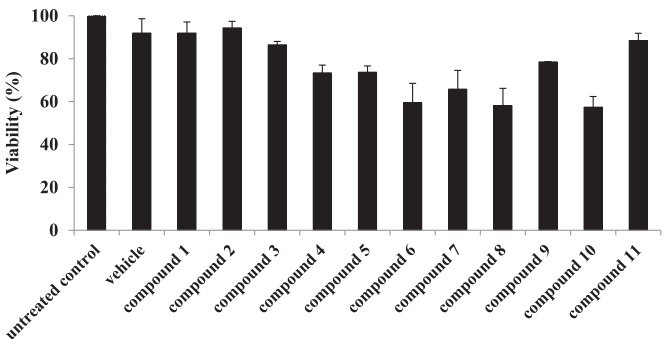


Fig. 2. Effect of rosmarinic acid (1) and analogs 2–11 on viability of MH-S macrophages. Cells were treated with the test compounds at 25 μM for 24 h, and the viability was examined by an MTT assay. Data are given as mean ± S.E.M. of independent experiments.

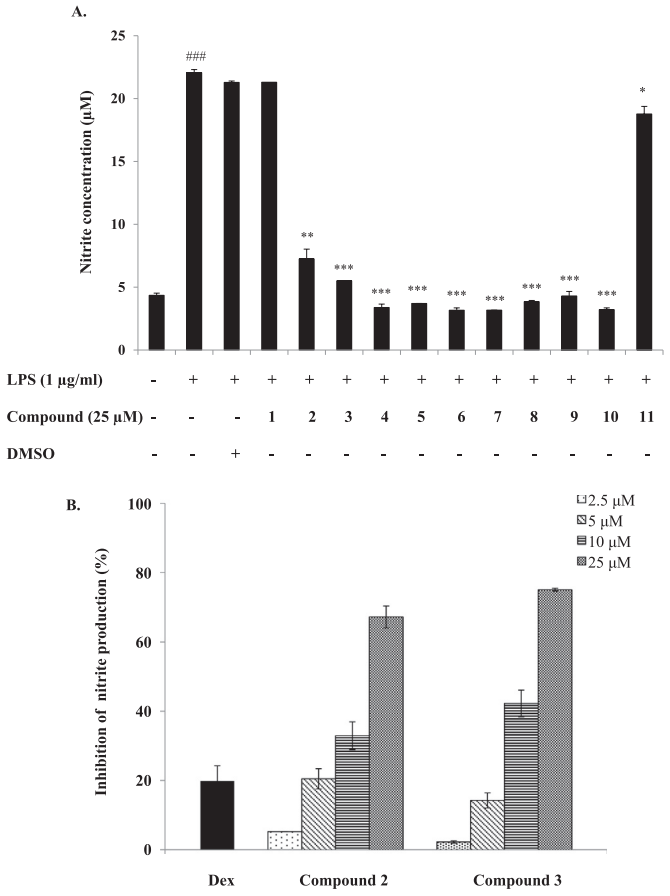


Fig. 3. Inhibition of rosmarinic acid (1) and analogs 2–11 on LPS-induced NO production in MH-S macrophages. (A) Initial screening. Cells were stimulated with LPS (1 μg/ml) in the presence or absence of the test compounds (25 μM) for 24 h. The concentration of nitrite in culture supernatant was determined by Griess assay. (B) NO inhibitory activity of compounds **2** and **3**. MH-S cells were stimulated with LPS (1 μg/ml) in the presence or absence of the test compounds at 2.5, 5, 10 and 25 μM or dexamethasone (Dex; 10 μM) for 24 h and nitrite concentration measured by Griess assay. Data are presented as mean ± S.E.M. of independent experiments. ### $P < 0.001$ compared with untreated group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with LPS-treated group.

inhibitory effects against LPS-induced NO release, and their IC_{50} values were determined. As shown in Fig. 3B, dose-dependent inhibition of LPS-induced NO release was caused by these two compounds, with the IC_{50} values of 12.42 and 15.09 μM for compounds **3** and **2**, respectively. At 10 μM, compound **3** inhibited NO production by 42.23% ± 3.86%; at the same concentration, dexamethasone only resulted in 19.89% ± 4.37% inhibition. Given the strong inhibitory activity of ethyl rosmarinate (**3**), this compound was therefore selected for subsequent experiments.

3.3. Effect of ethyl rosmarinate (3) on LPS-induced PGE_2 production in MH-S macrophages

As shown in Fig. 4, compound **3** strongly inhibited the production of PGE_2 in LPS-induced MH-S macrophages; the effect occurred in a dose-dependent manner. It was noted that the complete inhibition of PGE_2 production in LPS-treated MH-S cells was occurred after treatment at 25 μM. When expressed as a percentage inhibition, 10 μM compound **3** inhibited PGE_2 production by 90.31% ± 9.67%, whereas 10 μM dexamethasone caused 49.38% ± 14.97% inhibition.

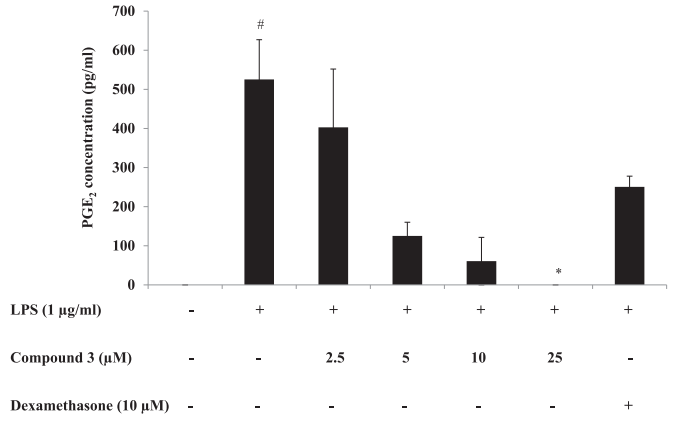


Fig. 4. Effect of ethyl rosmarinate (3) on LPS-induced PGE_2 production in MH-S macrophages. Cells were stimulated with LPS (1 μg/ml) in the presence or absence of compound **3** (2.5, 5, 10 and 25 μM) or dexamethasone (Dex; 10 μM) for 48 h. Concentrations of PGE_2 in culture supernatant were determined by enzyme-immunoassay. Data are presented as mean ± S.E.M. of independent experiments. # $P < 0.05$ compared with untreated group; * $P < 0.05$ compared with LPS-treated group.

3.4. Effects of ethyl rosmarinate (3) on iNOS and COX-2 mRNA and protein expression

In order to determine whether the inhibitory effects of compound **3** on NO and PGE_2 were related to the regulation of iNOS and COX-2 enzymes, the expression levels of these two enzymes were examined by RT-PCR and Western blot analysis. As presented in Fig. 5A, the mRNA level of iNOS was markedly increased after LPS treatment; this increase was significantly ($P < 0.01$) inhibited by treatment with compound **3**. Western blotting analysis also demonstrated that expression of iNOS protein was upregulated by exposure to LPS; similarly this increase was significantly inhibited as increasing concentrations of compound **3** were applied (Fig. 5B). At 25 μM, compound **3** reduced the expression of iNOS mRNA and protein to similar levels of unstimulated cells ($P > 0.05$). Furthermore, treatment with compound **3** inhibited the LPS-induced expression of COX-2 mRNA and protein in a dose-dependent manner (Fig. 5C and D). Unexpectedly, although the effect on COX-2 protein expression was not observed at 24 h (see Supplementary data), the reduction in LPS-stimulated COX-2 protein expression mediated by compound **3** was evident at 48 h, which suggested that the effect may be modulated by translational and/or post-translational regulation effects in the late stage.

3.5. Effect of ethyl rosmarinate (3) on NF-κB activation

To further investigate whether the inhibition of NO by compound **3** was mediated by the NF-κB activation pathway, we examined the expression level of p65, part of the NF-κB transcription factor, in the presence of compound **3** by using Western blotting. The results in Fig. 6 demonstrated that the expression level of p65 in the nuclear fraction increased dramatically after stimulation with LPS for 30 min compared with the untreated control. However, although the difference was not statistically significant, treatment with compound **3** decreased the expression level of p65 in the nucleus to a value close to the baseline in unstimulated MH-S macrophage cells ($P > 0.05$).

4. Discussion

Alveolar macrophages, which play a critical role in the pathogenesis of COPD, NO and PGE_2 are considered to be important biomarkers in this lung inflammatory disorder (Barnes, 2016). Elevated PGE_2 levels and significantly increased iNOS expression and activity in the lungs of patients with COPD have been well described, and this has been suggested to be associated with the development of the severe impairments

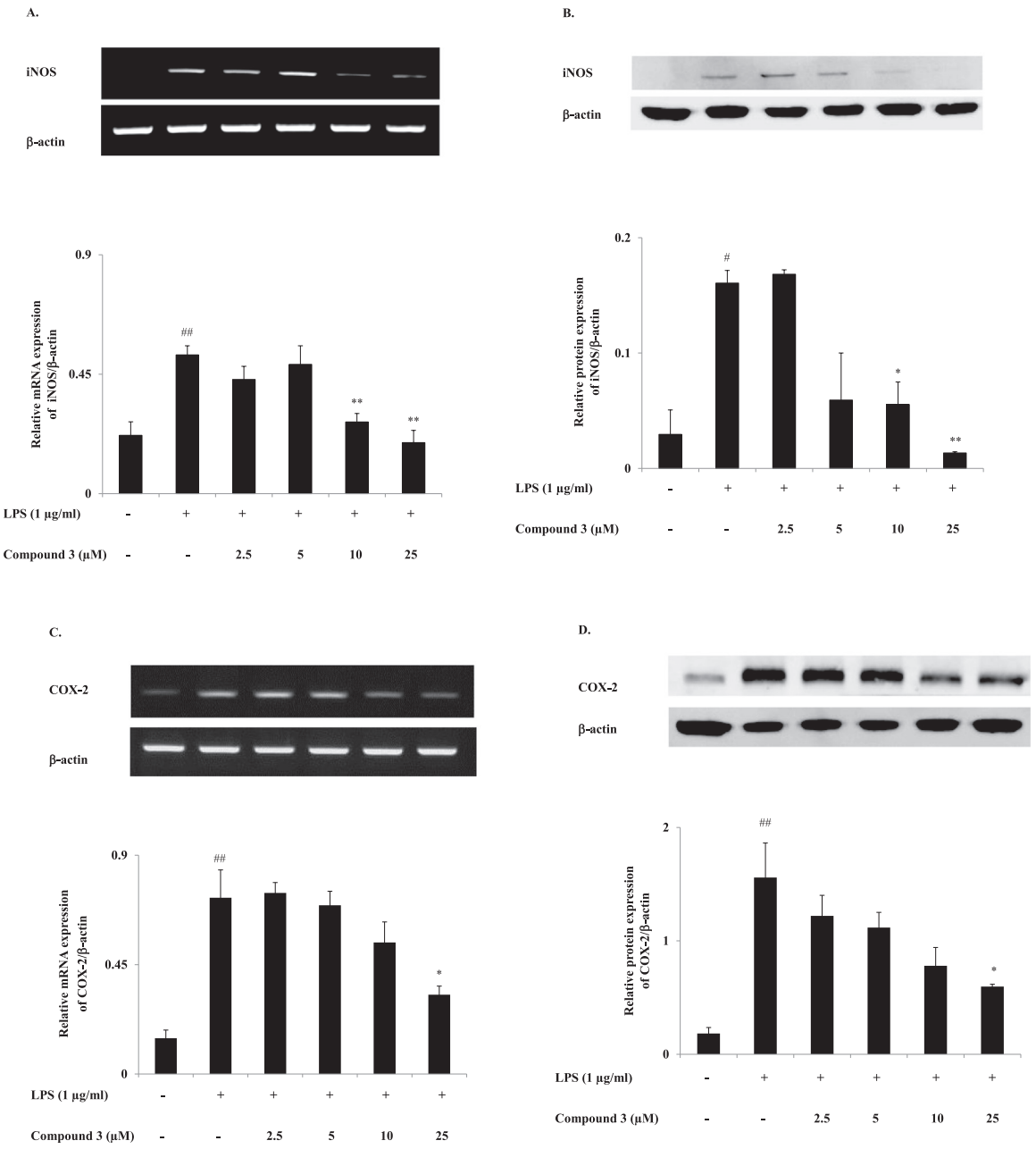


Fig. 5. Effects of ethyl rosmarinate (3) on iNOS and COX-2 mRNA and protein expression in LPS-induced MH-S macrophages. Cells were stimulated with LPS (1 μg/ml) in the presence or absence of compound **3** (2.5, 5, 10 and 25 μM) as indicated in Materials and Methods. Expressions of iNOS and COX-2 mRNA and protein were determined by RT-PCR and Western blotting analysis, respectively. Data are presented as mean ± S.E.M. of independent experiments. # $P < 0.05$, ## $P < 0.01$ compared with untreated group; * $P < 0.05$, ** $P < 0.01$ compared with LPS-treated group.

in COPD (Maestrelli et al., 2003; Brindicci et al., 2010). Owing to their influence on lung inflammation, alveolar macrophages are a good therapeutic target for airway inflammatory diseases, particularly COPD (Costa et al., 2015). In the present study, the anti-inflammatory potentials of rosmarinic acid (**1**) and its analogs **2–11** were investigated in an LPS-induced inflammatory model of alveolar macrophages. LPS was used to induce alveolar macrophage inflammation as this component of gram-negative bacterial cell wall is responsible for inducing pathological features of COPD (Ghorani et al., 2017). LPS also plays a significant role in the bacterial infection-induced exacerbation of COPD, which contributes to the development of the disease (Patel et al., 2002; Wright et al., 2008; Pera et al., 2011). Although cigarette smoke is the most important risk factor for COPD (Li et al., 2012), a large body of evidence has revealed that LPS is one of the major active components in cigarette smoke (Larsson et al., 2008). Recently, a methodological review determined that LPS was among the three most commonly used agents for the induction of COPD in an animal model that mimics the important features of COPD (Fehrenbach, 2006; Wright et al., 2008). Both *in vitro* and *in vivo* LPS-induced inflammation models have been well-accepted as valuable tools for the investigation of COPD (Chen et al., 2007; Gao et al., 2014; Ghorani et al., 2017). The results from this study clearly showed that, among the test compounds, ethyl rosmarinate (**3**) exhibited the most potent inhibitory action on NO production in LPS-induced MH-S cells and exhibited low cytotoxicity. Compound **3** also showed strong inhibitory activity against the production of PGE_2 in LPS-induced MH-S cells. In particular, the inhibitory potency of compound **3** against LPS-induced NO and PGE_2 release was approximately two-fold higher than that of the reference drug, dexamethasone (Ardestani et al., 2017). As compound **3** significantly inhibited both inflammatory mediators, it would therefore provide the most potent

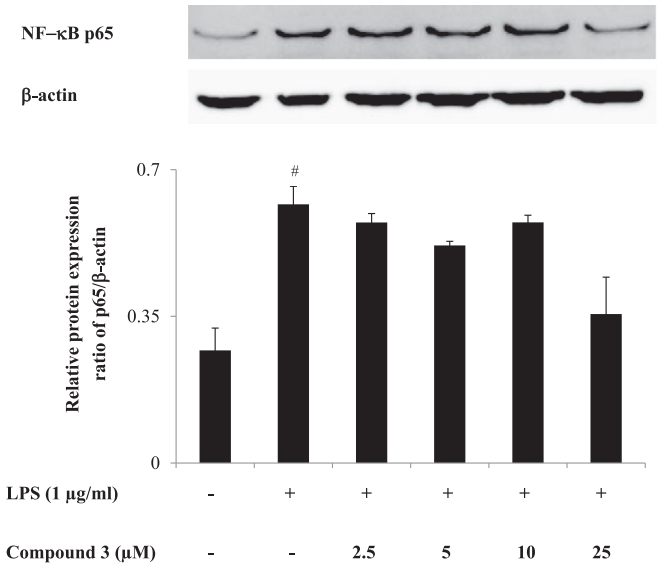


Fig. 6. Effect of ethyl rosmarinate (3) on NF-κB p65 activation in LPS- stimulated MH-S macrophages. Cells were treated with compound **3** at 2.5, 5, 10 and 25 μM and LPS 1 μg/ml. After 30 min incubation, the expression of NF-κB p65 in the nuclear fraction was determined by Western blotting. Results are presented as mean ± S.E.M. of independent experiments. # $P < 0.05$ compared with untreated group.

anti-inflammatory effects. Our results strongly indicated the potential of compound **3** as a novel anti-inflammatory agent for the treatment of macrophage-mediated inflammatory lung diseases, such as COPD.

NO and PGE_2 are synthesized by inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively (Kanwar et al., 2009; Gao et al., 2014). To explore the anti-inflammatory mechanisms, the effects of compound **3** on the LPS-induced iNOS and COX-2 mRNA and protein expression were investigated. Our results demonstrated that compound **3** significantly decreased both the LPS-induced mRNA and protein up-regulation of iNOS and COX-2 in MH-S macrophages (Fig. 5), and such decreases were correlated with the accumulation of NO (Fig. 3) and PGE_2 (Fig. 4). As NF-κB p65 is critical for the induction of iNOS and COX-2 expression in LPS-induced alveolar macrophages (Li et al., 2002), the effect of compound **3** on the LPS-induced activation of NF-κB p65 was assessed further. Given that NF-κB pathway is central to the pathogenesis of COPD, this pathway offers an excellent therapeutic target for the treatment of inflammatory diseases, including COPD (Edwards et al., 2009). We found that the decrease in the p65 expression level in the nucleus after compound **3** treatment (Fig. 6) was noticeably reduced to a level comparable with that of unstimulated alveolar macrophages. Although other pathways (e.g., AP-1 and IRF-3) may be involved and cannot be excluded, all our results suggested that compound **3** inhibited NO and PGE_2 production by decreasing the expression of iNOS and COX-2 mRNA and protein, at least partially through the downregulation of the NF-κB p65 activation pathway.

Through the investigation of anti-inflammatory activities by using a series of rosmarinic acid (**1**) and analogs **2–11** with different chemical structures, a relationship between the structure and the inhibitory activity could be drawn from this study. We found that although a natural rosmarinic acid (**1**) exerted no inhibitory activity, its methyl ester analog (compound **2**), which is also a natural compound, exhibited strong activity. The sharp increase in the inhibitory activity of NO production in going from the free carboxylic acid function to the methyl ester function has prompted us to investigate whether larger alkyl groups would give rise to analog(s) with higher inhibitory activity and a number of alkyl ester analogs, compounds **3–11**, were therefore synthesized. Ethyl rosmarinate (**3**) is the first synthetic analog of the series. This compound showed markedly stronger inhibitory activity than the parent compound **1**. An improvement of vasorelaxant activity of natural rosmarinic acid (**1**) after the structural modification to ethyl rosmarinate (**3**) has also been reported (Wicha et al., 2015). Although higher alkyl ester analogs **4–10** displayed strong inhibitory activities, such modifications led to cellular cytotoxicity, which would limit the future therapeutic applications of these compounds. To investigate whether the introduction of polar functionality to the ethyl ester group would give analog with higher inhibitory activity, the corresponding hydroxyl analog, compound **11**, was synthesized. Unexpectedly, a sharp decrease in inhibitory activity was observed, which suggested that an additional hydroxyl group abolished the activity. Collectively, these findings indicated the importance of hydrophobic ester group and the ethyl (CH_2CH_3) group seems to exert the most potent inhibitory activity against LPS-induced alveolar macrophage inflammation and its presence produced low toxicity.

5. Conclusion

This study demonstrated for the first time that ethyl rosmarinate (**3**), a structurally modified rosmarinic acid (**1**), exerted potent inhibitory activity against lung inflammation. This compound strongly inhibited NO and PGE_2 production in LPS-induced alveolar macrophages, without the induction of cellular toxicity. The mechanism responsible for the inhibitory effect on NO and PGE_2 production was the suppression of iNOS and COX-2 expression, which occurred at least in part through the NF-κB activation pathway. As ethyl rosmarinate inhibited key inflammatory mediators, our findings provided scientific evidence to support this compound as a promising novel therapeutic agent for the treatment of macrophage-mediated lung inflammatory diseases. Nevertheless, future investigations in an *in vivo* COPD model are required to support the future therapeutic applications of this compound.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2018.01.042.

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