available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Rengyolone inhibits inducible nitric oxide synthase expression and nitric oxide production by down-regulation of NF-κB and p38 MAP kinase activity in LPS-stimulated RAW 264.7 cells

Jin Hee Kim [a], *Dong Hyun Kim* [b], *Seung Hwa Baek* [a], *Ho Jae Lee* [a], *Mee Ree Kim* [c], *Ho Jeong Kwon* [d], *Choong-Hwan Lee* [a,*]

[a] *Immune Modulator Research Laboratory, Korea Research Institute of Bioscience and Biotechnology, 52 Eoun-dong, Yuseong, Daejeon 305-333, Republic of Korea*
[b] *Integrity Biosolution LLC, 820 Calle Plano Camarillo, CA 93012, USA*
[c] *Department of Food and Nutrition, Chungnam National University, 220 Gung-dong, Yusong, Daejeon 305-764, Republic of Korea*
[d] *Department of Biotechnology, Yonsei University, 134 Sinchon-dong, Seodaemun, Seoul 120-749, Republic of Korea*

ARTICLE INFO

Article history:
Received 5 October 2005
Accepted 23 December 2005

Keywords:
Rengyolone
Forsythia koreana
Nitric oxide
Inducible nitric oxide
Nuclear factor-κB
p38 MAPK

ABSTRACT

Nitric oxide (NO) is recognized as a mediator and regulator of inflammatory responses. Rengyolone, a cyclohexylethanoid isolated from the fruits of *Forsythia koreana*, exhibits anti-inflammatory activity with unknown mechanism. In this study, we found that rengyolone has a strong inhibitory effect on the production of nitric oxide (NO) and tumor necrosis factor-α (TNF-α). Rengyolone also inhibited inducible nitric oxide synthase (iNOS) gene expression and cyclooxygenase 2 (COX-2) by lipopolysaccharide (LPS). In order to explore the mechanism responsible for the inhibition of iNOS gene expression by rengyolone, we investigated its effect on LPS-induced nuclear factor-κB (NF-κB) activation. The LPS-induced DNA binding activity of NF-κB was significantly inhibited by rengyolone, and this effect was mediated through inhibition of the degradation of inhibitory factor-κBα and phosphorylation of p38 MAP kinase. Furthermore, rengyolone suppressed the expression of ICE protein in IL-1β-treated D10S cells. Taken together, these results suggest that rengyolone attenuates the inflammation through inhibition of NO production and iNOS expression by blockade of NF-κB and p38 MAPK activation in LPS-stimulated RAW 264.7 cells.

1. Introduction

Nitric oxide (NO) is a short-lived free radical that mediates many biological functions, including neurotransmission, vascular homeostasis, and host defense [1,2]. Production of NO is controlled by nitric oxide synthase (NOS), which converts L-arginine to L-citrulline [3]. Three types of NOS have been identified: endothelial NOS (eNOS), neutral NOS (nNOS), and inducible NOS (iNOS) [4]. Most importantly, iNOS is highly expressed in lipopolysaccaride (LPS)-activated macrophages, and this contributes to the pathogenesis of septic shock [5]. In some cases, the induction of iNOS by other stimuli leads to organ destruction in some inflammatory [6] and autoimmune diseases [7]. Thus, the inhibition of NO production by blocking

* *Corresponding author at*: Immune Modulator Research Laboratory, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yuseong, Daejeon 305-333, Republic of Korea. Tel.: +82 42 860 4294; fax: +82 42 860 4595.
E-mail address: chlee@kribb.re.kr (C.-H. Lee).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2005.12.031

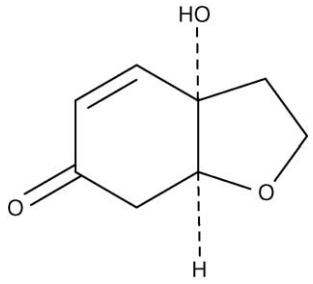

Fig. 1 – Chemical structure of rengyolone.

iNOS expression is an important target in the treatment of inflammatory diseases.

Expression of the iNOS gene in macrophages is regulated mainly at the transcriptional level, particularly by NF-κB [8]. In unstimulated cells, NF-κB is constitutively localized in the cytosol as a homodimer or heterodimer and is associated with inhibitory IκB protein (IκB). Activation of NF-κB results in phosphorylation, ubiquitination, and proteasome-mediated degradation of the IκB proteins, followed by the translocation of NF-κB to the nucleus and induction of gene transcription through binding to the *cis*-acting κB element [9]. Moreover, three well-defined mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH_2-terminal kinase (JNK), have been implicated in the transcriptional regulation of iNOS gene. Also, the specific MAPKs inhibitors suppress the expression of the iNOS gene [10–12].

In our ongoing study to discover new anti-inflammatory agent, rengyolone (Fig. 1), a cyclohexylethanoid isolated from the fruits of *Forsythia koreana*, was found to inhibit NO production in LPS-induced RAW 264.7 cells. The fruits of *F. koreana* are known to exhibit antibacterial, anti-inflammatory, and diuretic activities [13,14]. Moreover, rengyolone is reported to be responsible for their antibacterial activity [15]. However, how rengyolone can exhibit anti-inflammatory activity has not yet been uncovered. In this study, we demonstrate that rengyolone inhibited LPS-induced NO production and iNOS expression in RAW 264.7 cells, apparently through inhibiting of p38 MAPK activation and the blocking of NF-κB binding to the iNOS promotor, resulting in the inhibition iNOS induction.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. LPS used in this study was from *Salmonella typhosa* (Sigma). Antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Fetal bovine serum was from Gibco BRL Life Technology (Rockville, MD). Rengyolone was isolated from the fruits of *F. koreana*. Briefly, dried medicinal plants (1 kg) were extracted with MeOH and the extract was concentrated in vacuo, and then extracted three times with an equal volume of EtOAc. The EtOAc extract on concentration left a dark syrup (25 g), which was chromatographed on a column of silica gel with $CHCl_3$ and MeOH mixtures increasing polarity. The active fractions were further purified by Sephadex LH-20 column chromatography using as solvent system MeOH–H_2O (5:5). Final purifications were conducted by high-performance liquid chromatography using an ODS column (YMC C_{18}) with an acetonitrile–H_2O gradient solvent system and resulted in pure compound (7.1 mg). Based on electron spray ionization-mass spectrometry, ^1H NMR and ^{13}C NMR spectral data, the purified compound was verified as rengyolone ($C_8H_{10}O_3$; M_W 154.02 kDa) [16].

2.2. Cell culture

RAW 264.7 cells (ATCC TIB71) were purchased from American Type Culture Collection (Bethesda, MD, USA). Cells were grown in DMEM (Gibco BRL, USA) supplemented with 10% fetal bovine serum (Gibco BRL, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin. Non-adherent cells were removed by repeated washing after 2 h incubation at 37 °C. D10S cells, a subclone of the murine D10G.1.helper T-cell, were used for the cell lines of ICE activity. The cells were maintained in a RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum and 10% (v/v) mouse-conditioned medium, as described in [17,18]. Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Cell viability assay

Cell viability was evaluated by a 3-(4,5-dimethylthiazol-1)-5-(3-carboxymeth-oxyphenyl)-2H-tetrazolium (MTS) assay [19]. In the MTS assay, the cell suspension was plated (100 μl) in a 96-well microculture plate. After seeding, various concentrations of the compound were added to the plate and incubated for 24 h. MTS/PMS solution was prepared by mixing 25 μl phenazinemethosulfate (PMS) (1.53 mg/ml in PBS) for every 975 μl MTS (1.71 mg/ml in PBS). Finally, 50 μl of MTS/PMS solution was added to each well and incubated for 1–3 h. The absorbance of formazan at 490 nm was measured directly from the 96-well assay plates without additional processing.

2.4. Nitrite quantification

Nitrite accumulation was used as an indicator of NO production in the medium as previously described [20]. Cells were plated at 5×10^5 cells/ml in 96-well culture plates and stimulated with rengyolone or LPS for 24 h. The isolated supernatants in cultured macrophages were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. Using $NaNO_2$ to generate a standard curve, the concentration of nitrite was measured by OD reading at 540 nm.

2.5. Enzyme-linked immunosorbent assay of TNF-α

The inhibitory effect of rengyolone on TNF-α production was determined as described by Cho et al. [21]. The final concentration of the vehicle was under 0.05% in the culture medium. After pre-incubation of RAW 264.7 cells for 24 h in 24-well plates, LPS (1 μg/ml) and various concentrations of rengyolone were added to the wells. Supernatants were

assayed for TNF-α content using a mouse TNF-α ELISA kit (Amersham, Little Chalfont, Buckinghamshire, UK).

2.6. Western blot analysis

Lysates (20 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk for 1 h and washed with 0.05% TBST (TBS containing 0.05% Tween 20). The membranes were then incubated for 2 h with antibody for iNOS (Upstate Biotechnology, Inc., Lake Placid, NY, USA), I-κBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ICE (Sigma, St. Louis, MO, USA), phosphorylated ERK, or ERK, phosphorylated SAPK/JNK, or SAPK/JNK, phosphorylated p38, or p38 (Cell Signaling Technology, Beverly, MA, USA). Immunoreactive bands were then detected by incubation with conjugates of anti-rabbit or anti-mouse IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham-Pharmacia Biotech, San Francisco, CA, USA) followed by exposure to X-ray film.

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

Total RNA was isolated using TRIZOL™ Reagent (Molecular Research Center, Cincinnati, OH, USA). The sequences used were as follows: iNOS, sense: 5′-CTGC-AGCACTTGGATCAG-GAACCTG-3′, antisense: 5′-GGGAGTAGCCTGTGTGCA-CCTG-GAA-3′, and β-actin, sense: 5′-TGGAATCCTGTGGCATCCA-TGAAAC-3′, antisense: 5′-TAAAACGCAGCTCAGTAACAGTCC-G-3′. Equal amounts of RNA were reverse-transcribed into cDNA using oligo(dT)$_{16}$ primers. For PCR, samples were heated to 94 °C for 3 min and cycled 30 times at 94 °C for 30 s, 55 °C for 30 s, and 94 °C for 1 min, after which an additional extension step at 72 °C for 5 min was included. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME, USA) followed by staining with ethidium bromide. The iNOS and β-actin primers produce amplified products at 496 and 430 bp, respectively.

2.8. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as previously described [22]. The oligonucleotide sequences for NF-κB were: 5′-GATCTCA-GAGGGGACTTTCCGAGAGA-3′. The double-stranded deoxyoligonucleotides were end-labeled with [γ-^{32}P]-ATP. Nuclear extracts (5 μg) were incubated with poly(dI–dC) and the ^{32}P-labeled DNA probe in binding buffer (100 mM KCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml of aprotinin and 1 μg/ml of leupeptin) for 10 min. DNA binding activity was investigated using the free probe on a 4.8% polyacrylamide gel in 0.5× TBE buffer (44.5 mM Tris, 44.5 mM boric acid and 1 mM EDTA). Following electrophoresis, the gel was dried and subjected to autoradiography.

2.9. Interleukin-1β converting enzyme (ICE) production

D10S cells, a subclone of the murine D10G.1.helper T-cell, which is highly responsive to IL-1, were kindly provided by Dr. Charles A. Dinarello, University of Colorado Health Sciences Center (Denver, CO) [17]. D10S cells in RPMI supplemented with 5% FBS and 10% mouse-conditioned medium were harvested and washed twice with cold PBS and cultured at a concentration of 2.5×10^4 cells/ml in RPMI containing 5% FBS. The cultures were treated with various concentrations of the compound and stimulated with 2 ng/ml of IL-1β. The cells were incubated for 36 h at 37 °C in a 5% CO_2 air atmosphere. After observing the cells under a microscope, the ICE activity was then estimated from the cell lysate using N-acetyl-Tyr-Val-Ala-Asp-(7-amino-4-trifluoro methylcoumarin) (Ac-YVAD-AFC) as a substrate. The cells were lysed with a TTE buffer (10 mM Tris–HCl, 0.5% Triton X100, 10 mM EDTA, pH 8.0) and kept on ice for 30 min, and then centrifuged. The enzyme reactions were performed in a buffer (100 mM HEPES, 10 mM DTT, 10% sucrose, 0.1% CHAPS, 0.1% BSA) supplemented with 100 μM of Ac-YVAD-AFC at 37 °C for 4 h. The released fluorescent was measured on a spectrofluorimeter (Perkin-Elmer LS50B) [23]. The excitation and emission wavelengths were 400 and 505 nm, respectively.

3. Results

3.1. Inhibitory effect of rengyolone on the production of NO and TNF-α in LPS-stimulated RAW 264.7 cells

In order to investigate the effect of rengyolone on NO production, we measured the accumulation of nitrite in culture media. NO production was monitored in RAW 264.7 cells stimulated by LPS in the presence or absence of rengyolone for 24 h. The basal level of nitrite in unstimulated cells was less than 1 nmol/10^6 cells (Fig. 2A). Upon treatment with rengyolone (10, 20, 40, 80, and 160 μM), the nitrite production by RAW 264.7 cells was inhibited in a dose-dependent manner (31, 25, 18, 8, and 4 nmol/10^6 cells, respectively; Fig. 2A).

TNF-α is the principle mediator of response to LPS and may play a role in the innate immune response [24]. Thus, we studied the effect of rengyolone on TNF-α production. Production of TNF-α was measured in a medium of RAW 264.7 cells cultured with LPS (1 μg/ml) in the presence or absence of rengyolone for 6 h. Rengyolone treatment showed dose-dependent inhibition of TNF-α production in LPS-induced RAW 264.7 cells (Fig. 2B). Examination of the cytotoxicity of rengyolone in RAW 264.7 macrophages by MTS assay indicated that, even at 160 μM, rengyolone did not affect the viability of RAW 264.7 cells (data not shown). Therefore, inhibition of LPS-stimulated nitrite and TNF-α production by rengyolone was not the consequence of a cytotoxic effect on these cells.

3.2. Inhibitory effect of rengyolone on iNOS protein and mRNA expression in LPS-stimulated RAW 264.7 cells

Several studies have demonstrated that induction of iNOS produces a large amount of NO during endotoxemia and under inflammatory conditions [25,26]. In view of the involvement of iNOS in the inflammatory process, we monitored iNOS gene expression in macrophages exposed to rengyolone. The effect

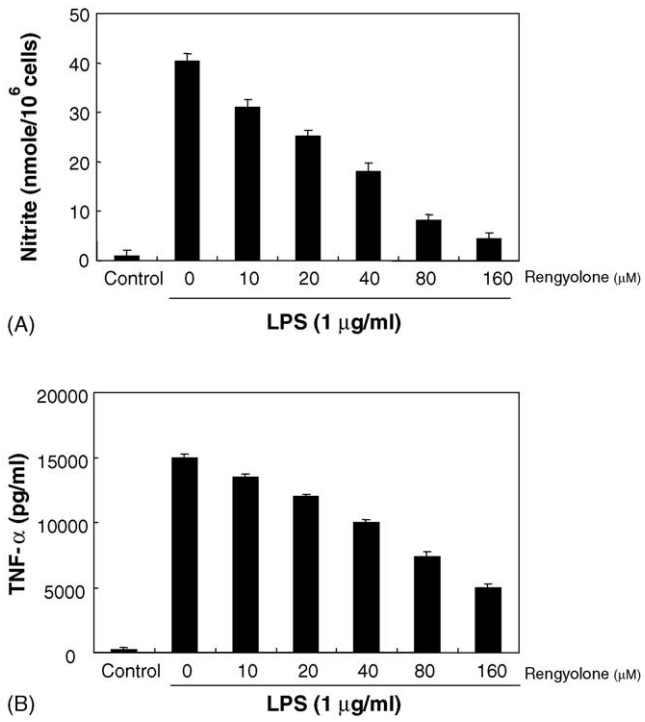

Fig. 2 – Inhibition of nitrite and TNF-α production by rengyolone in LPS-stimulated RAW 264.7 cells. (A) RAW 264.7 cells were pretreated with the indicated concentrations of rengyolone for 1 h before being incubated with LPS (1 μg/ml) for 24 h. Control cells were incubated with the vehicle alone. (B) Production of TNF-α was measured in a medium of RAW 264.7 cells cultured with LPS (1 μg/ml) in the presence or absence of rengyolone for 6 h. Control cells were incubated with the vehicle alone. Each column shows the mean ± S.D. of triplicate determinations.

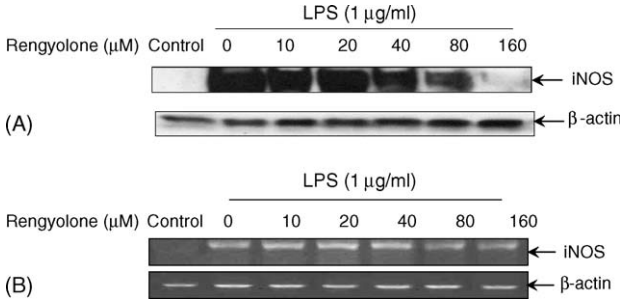

Fig. 3 – Inhibition of iNOS gene expression by rengyolone in LPS-stimulated RAW 264.7 cells. (A) Inhibition of iNOS protein expression by rengyolone. The level of iNOS protein was monitored 24 h after treatment of cells with LPS (1 μg/ml), with or without rengyolone. Cell lysates were then prepared and subjected to immunoblotting using an antibody specific for murine iNOS. (B) The effects of rengyolone on the iNOS mRNA level. RAW 264.7 cells were pretreated with the vehicle or indicated concentrations of rengyolone for 1 h before incubation with LPS (1 μg/ml) for 6 h. Total RNAs were isolated and iNOS mRNA expression was determined by RT-PCR.

of rengyolone on iNOS protein expression was examined by Western blot analysis. As shown in Fig. 3A, expression of the iNOS protein was barely detectable in unstimulated cells, but markedly increased 24 h after LPS (1 μg/ml) treatment. Consistent with previous results, treatment with rengyolone showed dose-dependent inhibition of iNOS protein expression in LPS-stimulated RAW 264.7 cells.

In order to assess the effect of rengyolone on iNOS mRNA expression, we measured mRNA levels by RT-PCR. The expression of iNOS mRNA was barely detectable in unstimulated cells. However, RAW 264.7 cells expressed high levels of iNOS mRNA when stimulated with LPS (1 μg/ml) for 6 h. Furthermore, rengyolone inhibited this LPS-induced iNOS mRNA production in a dose-dependent manner (Fig. 3B). RT-PCR and Western blot analyses revealed that iNOS expression by rengyolone was in parallel with comparable inhibition of NO production.

3.3. *Inhibitiory effect of rengyolone on the COX-2 protein expression in LPS-stimulated RAW 264.7 cells*

COX-2 is induced several stimuli such as LPS, certain serum factors, cytokines, and growth factors, and is predominantly expressed at sites of inflammation [27,28]. Therefore, there is increasing interest in the applicability of COX-2 inhibitors. We evaluated the effect of rengyolone on LPS-inducible COX-2 protein expression in RAW 264.7 cells. The expression of COX-2 protein was monitored in RAW 264.7 cells exposed to LPS (1 μg/ml) for 24 h. Rengyolone effectively inhibited the induction of COX-2 by LPS (Fig. 4).

3.4. *Effect of rengyolone on LPS-induced NF-κB activation in RAW 264.7 cells*

Activation of NF-κB is necessary for induction of the iNOS gene [8]. We therefore examined the effect of rengyolone on the activation of NF-κB. LPS treatment caused a significant increase in the DNA binding activity of NF-κB, as determined by an electrophoretic mobility shift assay (Fig. 5A). In the presence of rengyolone, LPS-induced NF-κB binding was markedly suppressed in a dose-dependent manner (Fig. 5A). Since p65 is the major component of NF-κB activated by LPS in macrophages, we next determined the levels of p65 in the nuclear extract by a Western blot analysis (Fig. 5B). RAW 264.7 cells were incubated with LPS in the presence or absence of rengyolone for 1 h. The LPS-induced nuclear translocation of

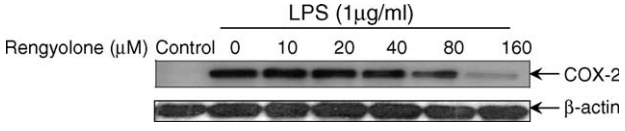

Fig. 4 – Inhibition of LPS-inducible COX-2 protein expression by rengyolone in RAW 264.7 cells. COX-2 protein expression was measured in RAW 264.7 cells cultured with LPS (1 μg/ml), with or without various concentrations of rengyolone for 24 h. Control cells were incubated with the vehicle alone.

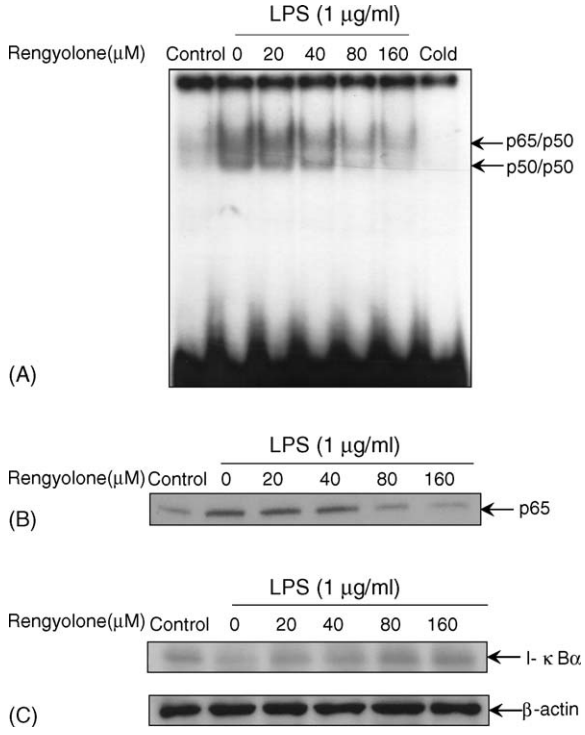

Fig. 5 – Inhibition of LPS-inducible NF-κB activation and I-κBα degradation by rengyolone in RAW 264.7 cells. (A) Nuclear extracts (5 μg) were prepared and analyzed for DNA binding activity of NF-κB using an electrophoretic mobility shift assay. RAW 264.7 cells were pretreated with the vehicle or indicated concentrations of rengyolone for 1 h before stimulation with LPS (1 μg/ml) for another 1 h. The result shown is representative of three independent experiments. (B) The p65 subunit of NF-κB and β-actin in nuclear extracts was determined by a Western blot analysis. RAW 264.7 cells were treated with LPS (1 μg/ml) for 1 h and p65 protein was detected using anti-p65 antibody. LPS caused the p65 protein to migrate to the nucleus at 1 h. (C) RAW 264.7 cells were pretreated with the vehicle or indicated concentrations of rengyolone for 1 h before stimulation with LPS (1 μg/ml) for 10 min. The protein levels of I-κBα and β-actin in nuclear extracts was determined by a Western blot analysis.

p65 (Fig. 5B) and the NF-κB DNA binding activity were both inhibited by rengyolone in a dose-dependent manner similar to that of NF-κB binding.

The nuclear translocation and DNA binding of the NF-κB transcription factor are preceded by the degradation of inhibitory factor-κBα (I-κBα) [29]. In order to determine whether the inhibition of NF-κB DNA binding by rengyolone is related to I-κBα degradation, cytoplasmic levels of I-κBα were examined by a Western blot analysis. Pretreatment of RAW 264.7 cells with rengyolone blocked LPS-induced I-κBα degradation (Fig. 5C). Since NF-κB is activated by I-κBα degradation following phosphorylation of I-κBα at serine residues [30], the recovery of I-κBα protein in RAW 264.7 cells provides strong evidence that rengyolone inhibited the activation of NF-κB.

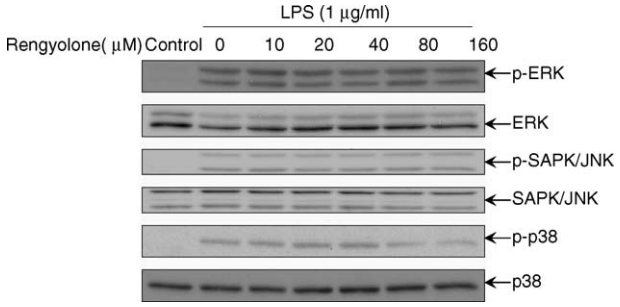

Fig. 6 – Effect of rengyolone on LPS-induced phosphorylation of ERK1/2, SAPK/JNK, and p38 MAP kinase in RAW 264.7 cells. RAW 264.7 cells were treated with the vehicle or indicated concentrations of rengyolone for 1 h before being incubated with LPS (1 μg/ml) for 30 min. The whole-cell lysates were analyzed by a Western blot analysis.

3.5. *Effect of rengyolone on the phosphorylation of MAPKs in LPS-stimulated RAW 264.7 cells*

The mitogen-activated protein (MAP) kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses. Moreover, they play a critical role for the activation of NF-κB [31]. In order to investigate whether the inhibition of NF-κB activation by rengyolone is mediated through the MAP kinase pathway, we examined the effect of rengyolone on the LPS-induced phosphorylation of ERK 1/2, SAPK/JNK, and p38 MAP kinase in RAW 264.7 cells using Western blot analyses. As shown in Fig. 6, rengyolone suppressed LPS-induced activation of p38 MAP kinase in a dose-dependent manner. The amount of non-phosphorylated p38 was unaffected by either LPS or rengyolone treatment. Rengyolone (80 and 160 μM) markedly inhibited p38 MAP kinase activation, while phosphorylation of the ERK 1/2 and SAPK/JNK MAP kinase was not affected by rengyolone treatment. These results suggest that phosphorylation of p38 is involved in the inhibitory effect of rengyolone on LPS-induced NF-κB binding in RAW 264.7 cells.

3.6. *Effect of rengyolone on interleukin-1β converting enzyme (ICE) production in D10S cells*

Caspase-1 is primarily involved in the processing of proinflammatory cytokines. The active form of caspase-1, the IL-1β converting enzyme (ICE), is frequently present in inflammatory diseases with a distribution similar to that of IL-1β, suggesting that caspase-1 is involved in the activation of proIL-1β to its active form [32]. In order to examine the effects of rengyolone on IL-1β-induced ICE production, D10S cells were treated with IL-1β (2 ng/ml) in the presence or absence of various concentrations of rengyolone. The compound showed the strongest inhibitory activity for ICE production in IL-1β-treated D10S cells with an IC_{50} value of 48.7 μM (Fig. 7A). Moreover, rengyolone suppressed the 45 kDa ICE protein expression induced by IL-1β (2 ng/ml) in a dose-dependent manner as shown in Fig. 7B. At 160 μM, rengyolone strongly

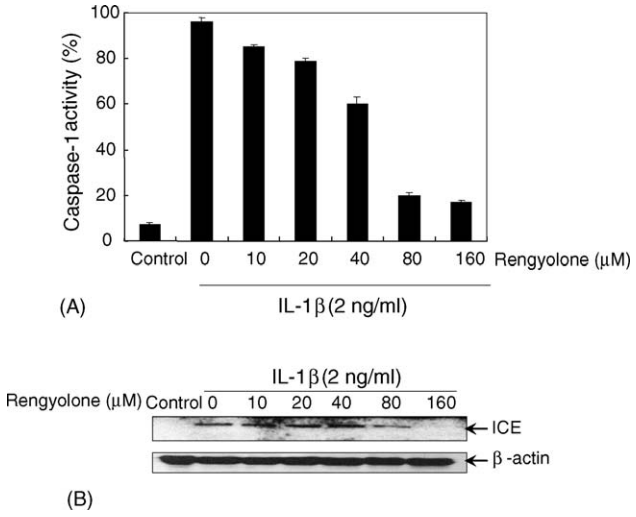

Fig. 7 – Effects of rengyolone on IL-1β-induced ICE in D10S cells. (A) Caspase-1 activity was decreased upon rengyolone treatment. D10S cells were incubated with or without IL-1β (2 ng/ml) in the presence of the indicated concentrations of rengyolone. (B) ICE protein expression was measured in D10S cells cultured with IL-1β (2 ng/ml), with or without various concentrations of rengyolone for 36 h. Control cells were incubated with the vehicle alone.

inhibited ICE production with no cellular toxicity, as determined by a MTS assay (data not shown). These results demonstrate that rengyolone may regulate ICE production and this activity can also contribute to the anti-inflammatory activity of the compound.

4. Discussion

Because the overproduction of NO can be harmful and result in various inflammatory and autoimmune diseases [33,34], pharmacological interference with the NO production cascade presents a promising strategy for therapeutic intervention in inflammatory disorders. In the present study, we demonstrated that rengyolone inhibits LPS-induced NO and TNF-α production. To explore the mechanism of inhibition of NO production in RAW 264.7 macrophages, the effect of rengyolone on the iNOS gene was examined. Rengyolone inhibited the expression of iNOS protein and mRNA, as assessed by Western bolt analyses and RT-PCR, respectively, in a similar dose-dependent manner. These results imply that rengyolone exerts its effect through the inhibition of the iNOS mRNA transcription step. The promoter of the iNOS gene is known to contain two transcriptional regions, an enhancer and a basal promoter [35]. There are a number of binding sites for transcription factors, including κB sites, located in both the enhancer and the basal promoter [8], and NF-κB is essential for LPS-mediated NO production. In unstimulated cells, NF-κB is present in the cytosol as a homodimer or a heterodimer and is linked to the inhibitory IκB protein. The activation of NF-κB results in phosphorylation, ubiquitination, and proteasome-mediated degradation of IκB proteins, followed by nuclear translocation and DNA binding of the NF-κB [9]. Our study indicate that rengyolone inhibited the LPS-induced DNA binding activity of NF-κB, as assessed in a nuclear extract with an EMSA assay. It also inhibited the nuclear translocation of p65 protein. In addition, rengyolone inhibited LPS-induced I-κBα degradation. Since NF-κB is activated by I-κBα degradation following phosphorylation of I-κBα at serine residues [30], the recovery of I-κBα protein in RAW 264.7 cells provides strong evidence that rengyolone inhibited the activation of NF-κB. Rengyolone is a α,β-unsaturated ketone compounds, which is known to react with nucleophiles, especially with SH groups in proteins, in Michael-type addition [36–39]. Therefore, NO production was monitored in RAW 264.7 cells stimulated by LPS in the presence or absence of rengyolone and DTT for 24 h. Upon treatment rengyolone co-incubated cells with DTT, the nitrite production by RAW 264.7 cells did not affected (data not shown).

There are at least three families of MAP kinases (ERK, JNK, and p38 MAPK) that exist in mammalian cells. These kinases play a critical role in the regulation of cell growth and differentiation, particularly in response to cytokines and stress [40]. Several studies have demonstrated the implication of MAPKs in LPS-induced iNOS expression [10–12,41] and the activation of NF-κB [42]. Cell stimulation induces a signaling cascade that leads to the activation of MAPKs via phosphorylation of both tyrosine and threonine residuces [43], which in turn induces a conformational change that exposes the active site for substrate binding. The result is that rengyolone inhibited LPS-induced activation of p38 MAP kinase in a dose-dependent manner.

A selective ICE inhibitor blocks both IL-1β and IFN-γ production induced by LPS from human mononuclear cells. Furthermore, caspase-1-deficent mice are defective in LPS-induced IFN-γ production [44]. As shown in Fig. 7, rengyolones can suppress IL-1β-induced ICE expression of D10S cells. These results imply that ICE plays a critical role in the regulation of various pro-inflammatory cytokines. Thus, specific ICE inhibitors would provide a new class of anti-inflammatory drugs with potent activity. As such, ICE becomes an even more attractive target in relation to the discovery of drugs to treat autoimmune inflammatory and other diseases in which these cytokines are implicated. Further studies will focus on the in vivo application of rengyolone for curative possibility in inflammatory-related diseases.

In summary, this study demonstrates that rengyolone inhibits LPS-induced NO production, TNF-α production, and iNOS gene expression in macrophages and that these effects are mediated by inhibition of the activity of NF-κB and the phosphorylation of p38. In view of the fact that NO plays an important role in mediating inflammatory responses, the inhibitory effect of rengyolone on iNOS gene expression might be responsible for its anti-inflammatory action.

Acknowledgement

This research was supported by a grant from the Plant Diversity Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology (MOST) of the Korean government.

REFERENCES

[1] Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109–42.
[2] Jaffrey SR, Snyder SH. Nitric oxide: a neural messenger. Annu Rev Cell Dev Biol 1995;11:417–40.
[3] MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol 1997; 15:323–50.
[4] Marletta MA. Nitric oxide synthase structure and mechanism. J Biol Chem 1993;268:12231–4.
[5] Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, et al. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 1992;256:225–8.
[6] McCartney-Francis N, Allen JB, Mizel DE, Albina JE, Xie QW, Nathan CF, et al. Suppression of arthritis by an inhibitor of nitric oxide synthase. J Exp Med 1993;178:749–54.
[7] Kleemann R, Rothe H, Kolb-Bachofen V, Xie QW, Nathan C, Martin S, et al. Transcription and translation of inducible nitric oxide synthase in the pancreas of prediabetic BB rats. FEBS Lett 1993;328:9–12.
[8] Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem 1994;269:4705–8.
[9] Karin M. The beginning of the end: I kappa B kinase (IKK) and NF-kappa B activation. J Biol Chem 1999;274:27339–42.
[10] Chen C, Chen YH, Lin WW. Involvement of p38 mitogen-activated protein kinase in lipopolysaccharide-induced iNOS and COX-2 expression in J774 macrophages. Immunology 1999;97:124–9.
[11] Chan ED, Riches DW. Potential role of the JNK/SAPK signal transduction pathway in the induction of iNOS by TNF-alpha. Biochem Biophys Res Commun 1998;253:790–6.
[12] Kim YH, Lee SH, Lee JY, Choi SW, Park JW, Kwon TK. Triptolide inhibits murine-inducible nitric oxide synthase expression by down-regulating lipopolysaccharide-induced activity of nuclear factor-kappa B and c-Jun NH_2-terminal kinase. Eur J Pharmacol 2004;494:1–9.
[13] Kim NY, Kang TH, Song EK, Pae HO, Chung HT, Kim YC. Inhibitory effects of butanol fraction of the aqueous extract of *Forsythia koreana* on the nitric oxide production by murine macrophage-like RAW 264.7 cells. J Ethnopharmacol 2000;73:323–7.
[14] Kitagawa S, Tsukamoto H, Hisada S, Nishibe S. Studies on the Chinese crude drug *Forsythiae fructus*. Chem Pharm Bull 1984;32:1209–13.
[15] Jensen SR, Franzyk H, Wallander E. Chemotaxonomy of the Oleaceae: iridoids as taxonomic markers. Phytochemistry 2002;60:213–31.
[16] Endo K, Seya K, Hikino H. Biogenesis-like transformation of salidroside to rengyol and its related cyclohexylethanoids of *Forsythia suspensa*. Tetrahedron 1989;45:3673–82.
[17] Orencole SF, Dinarello CA. Characterization of a subclone (D10S) of the D10.G4.1 helper T-cell line which proliferates to attomolar concentrations of interleukin-1 in the absence of mitogens. Cytokine 1989;1:14–22.
[18] Yoon DY, Dinarello CA. Antibodies to domains II and III of the IL-1 receptor accessory protein inhibit IL-1 beta activity but not binding: regulation of IL-1 responses is via type I receptor, not the accessory protein. J Immunol 1998;160:3170–9.
[19] Goodwin CJ, Holt SJ, Downes S, Marshall NJ. Microculture tetrazolium assays: a comparison between two new tetrazolium salts, XTT and MTS. J Immunol Meth 1995;179:95–103.
[20] Green LC, Ruiz de Luzuriaga K, Wagner DA, Rand W, Istfan N, Young VR, et al. Nitrate biosynthesis in man. Proc Natl Acad Sci USA 1981;78:7764–8.
[21] Cho JY, Park J, Yoo ES, Yoshikawa K, Baik KU, Lee J, et al. Inhibitory effect of lignans from the rhizomes of *Coptis japonica* var. dissecta on tumor necrosis factor-alpha production in lipopolysaccharide-stimulated RAW 264.7 cells. Arch Pharm Res 1998;21:12–6.
[22] Yoon YD, Han SB, Kang JS, Lee CW, Park SK, Lee HS, et al. Toll-like receptor 4-dependent activation of macrophages by polysaccharide isolated from the radix of *Platycodon grandiflorum*. Int Immunopharmacol 2003;3:1873–82.
[23] Gurtu V, Kain SR, Zhang G. Fluorometric and colorimetric detection of caspase activity associated with apoptosis. Anal Biochem 1997;251:98–102.
[24] Tracey KJ, Cerami A. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. Annu Rev Med 1994;45:491–503.
[25] Fischer LG, Horstman DJ, Hahnenkamp K, Kechner NE, Rich GF. Selective iNOS inhibition attenuates acetylcholine- and bradykinin-induced vasoconstriction in lipopolysaccharide-exposed rat lungs. Anesthesiology 1999;91:1724–32.
[26] Avontuur JA, Boomsma F, van den Meiracker AH, de Jong FH, Bruining HA. Endothelin-1 and blood pressure after inhibition of nitric oxide synthesis in human septic shock. Circulation 1999;99:271–5.
[27] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[28] Wadleigh DJ, Reddy ST, Kopp E, Ghosh S, Herschman HR. Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. J Biol Chem 2000;275:6259–66.
[29] Rice NR, Ernst MK. In vivo control of NF-kappa B activation by I kappa B alpha. EMBO J 1993;12: 4685–95.
[30] May MJ, Ghosh S. Signal transduction through NF-kappa B. Immunol Today 1998;19:80–8.
[31] Nakano H, Shindo M, Sakon S, Nishinaka S, Mihara M, Yagita H, et al. Differential regulation of I kappa B kinase alpha and beta by two upstream kinases, NF-kappa B-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc Natl Acad Sci USA 1998;95:3537–42.
[32] Thornberry NA, Molineaux SM. Interleukin-1 beta converting enzyme: a novel cysteine protease required for IL-1 beta production and implicated in programmed cell death. Protein Sci 1995;4:3–12.
[33] Southan GJ, Szabo C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. Biochem Pharmacol 1996;51:383–94.
[34] Guzik TJ, Korbut R, Adamek-Guzik T. Nitric oxide and superoxide in inflammation and immune regulation. J Physiol Pharmacol 2003;54:469–87.
[35] Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW, et al. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. Proc Natl Acad Sci USA 1993;90:9730–4.
[36] Lee SH, Lee SY, Son DJ, Lee H, Yoo HS, Song S, et al. Inhibitory effect of 2′-hydroxycinnamaldehyde on nitric oxide production through inhibition of NF-kappa B activation in RAW 264.7 cells. Biochem Pharmacol 2005;69:791–9.
[37] Ji C, Kozak KR, Marnett LJ. I kappa B kinase, a molecular target for inhibition by 4-hydroxy-2-nonenal. J Biol Chem 2001;276:18223–8.

[38] Moos PJ, Edes K, Mullally JE, Fitzpatrick FA. Curcumin impairs tumor suppressor p53 function in colon cancer cells. Carcinogenesis 2004;25:1611–7.
[39] Pan MH, Lin-Shiau SY, Lin JK. Comparative studies on the suppression of nitric oxide synthase by curcumin and its hydrogenated metabolites through down-regulation of I kappa B kinase and NF-kappa B activation in macrophages. Biochem Pharmacol 2000;60:1665–76.
[40] Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002;298:1911–2.
[41] Kim SH, Kim J, Sharma RP. Inhibition of p38 and ERK MAP kinases blocks endotoxin-induced nitric oxide production and differentially modulates cytokine expression. Pharmacol Res 2004;49:433–9.
[42] Carter AB, Knudtson KL, Monick MM, Hunninghake GW. The p38 mitogen-activated protein kinase is required for NF-kappa B-dependent gene expression. The role of TATA-binding protein (TBP). J Biol Chem 1999;274:30858–63.
[43] Kyriakis JM, Avruch J. Sounding the alarm: protein kinase cascades activated by stress and inflammation. J Biol Chem 1996;271:24313–6.
[44] Jang SI, Jeong SI, Kim KJ, Kim HJ, Yu HH, Park R, et al. Tanshinone IIA from *Salvia miltiorrhiza* inhibits inducible nitric oxide synthase expression and production of TNF-alpha, IL-1beta and IL-6 in activated RAW 264.7 cells. Planta Med 2003;69:1057–9.