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SUPPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE AND CYCLOOXYGENASE-2 EXPRESSION IN RAW 264.7 MACROPHAGES BY SESQUITERPENE LACTONES

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The molecular mechanism underlying the suppression of lipopolysaccharide (LPS)/interferon-γ (IFN-γ)-induced nitric oxide (NO) and prostaglandin (PG) E_2 production was investigated in RAW 264.7 macrophages treated with sesquiterpene lactones, zaluzanin-C and estafiatone, isolated from Ainsliaea. Zaluzanin-C and estafiatone decreased NO production in LPS/IFN-γ-stimulated RAW 264.7 macrophages with an IC50 of about 6.61 μM and 3.80 μM, respectively. In addition, these compounds inhibited the synthesis of PGE_2 in LPS/IFN-γ-treated RAW 264.7 macrophages. Furthermore, treatment with zaluzanin-C and estafiatone resulted in a decrease in inducible No Synthase (iNOS) and Cyclooxygenase-2 (COX-2) protein and mRNA expression levels. Zaluzanin-C and estafiatone inhibited nuclear factor-κB (NF-κB) activation, a transcription factor necessary for iNOS and COX-2 expression in response to LPS/IFN-γ. This effect was accompanied by parallel reduction of phosphorylation and degradation of inhibitor of κB (IkB). In addition, these effects were completely blocked by treatment with cysteine, indicating that the inhibitory effect of zaluzanin-C and estafiatone might be mediated by alkylation of either NF-κB itself or an upstream molecule of NF-κB. These results demonstrate that the suppression of NF-κB activation by zaluzanin-C and estafiatone might be attributed to inhibition of nuclear translocation of NF-κB resulting from blockade of the degradation of IκB, leading to suppression of the expression of iNOS and COX-2, which play important roles in inflammatory signaling pathways.

Nitric oxide (NO), prostaglandins (PG) and their associated enzymes NO synthases (NOS) and cyclooxygenases (COX, specifically COX-2) have been implicated in the development of inflammation (Nathan, 1997; Sautebin, 2000). Three isoforms of NOS exist: constitutively expressed neuronal NOS (nNOS) and endothelial NOS (eNOS), which are both Ca^{2+}-calmodulin-dependent, and an inducible isoform (iNOS), which is Ca^{2+}-calmodulin-independent (Michel et al., 1995; Rao, 2000). Two isoforms of COX exist, constitutively expressed COX-1 and an inducible isoform COX-2 (Smith et al.,

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Sang Gyu Shin and Jae Ku Kang contributed equally to this work.

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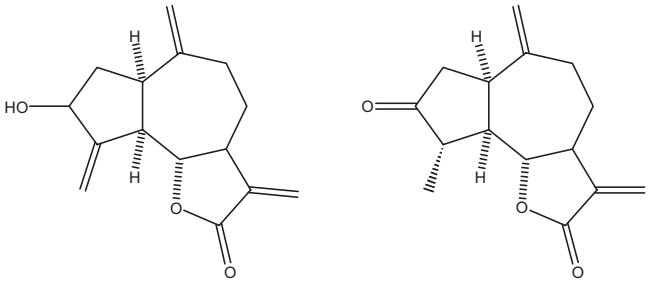

FIGURE 1. Chemical structure of zaluzanin-C and estafiatone isolated from *Ainsliaea acerifolia*.

1996). iNOS and COX-2 are upregulated in response to inflammatory and proinflammatory mediators (Fu et al., 1990; Moncada et al., 1991; MacMicking et al., 1997; Sautebin et al., 1998), and their products were shown to be important mediators of acute and chronic inflammation.

Nuclear factor-κB (NF-κB) is a transcription factor acting as a central mediator of the human immune response, and regulates the transcription of various inflammatory cytokines such as interleukin (IL)-1, IL-2, IL-6, IL-8, and tumor necrosis factor (TNF)-α as well as genes encoding cell adhesion molecules, immunoreceptors, hematopoietic growth factors, and growth factor receptors (Baeuerle & Henkel, 1994; Kang et al., 2000, 2001). In addition, NF-κB response elements have been demonstrated to be on the promoter regions of iNOS and COX-2 (Barnes & Karin, 1997; Crofford et al., 1997), which are involved in inflammatory processes through producing NO and PGs, respectively. Therefore, NF-kB activation cascade has attracted much attention as a potential target for novel anti-inflammatory strategies.

Thus active compounds from many traditional herbal medicines were screened using iNOS and COX-2 expression, and NF-kB activation as a molecular target. Two sesquiterpene lactones, zaluzanin-C and estafiatone (Figure 1), were isolated from *Ainsliaea acerifolia* (A. acerifolia) which has been used as antipyretic, pain-relief, and anti-inflammatory traditional herbal medicine in Korea. These agents significantly inhibited NO and PGE_2 production in RAW 264.7 macrophages in preliminary experiments. This observation prompted us to elucidate inhibitory mechanism of NO and PGE_2 production by these sesquiterpene lactones, zaluzanin-C and estafiatone, by investigating the transcriptional regulation of iNOS and COX-2 and the NF-κB activation cascade in RAW 264.7 macrophages.

MATERIALS AND METHODS

Reagents

Zaluzanin-C and estafiatone (Figure 1) were extracted from *A. acerifolia* and the structural identity of these compounds was determined spectroscopically (^{1}H

and ^{13}C nuclear magnetic resonance [NMR], infrared [IR], mass spectrometry [MS]) as described previously (Han et al., 2001). Dulbecco's modified Eagle's medium (DMEM) with high glucose and other reagents for cell culture were obtained from Gibco BRL Life Biotechnologies (Grand Island, NY). ^{32}P-labeled dATP (3000 Ci/mmol) was from NEN Life Science Products (Boston). Anti-mouse iNOS and COX-2 antibodies were obtained from Transduction Laboratories (Lexington, KY). Rabbit polyclonal IκB-α and phospho-IκB-α antibody were from New England BioLabs (USA). Lipopolysaccharide (LPS) (from *Escherichia coli*, 011:B4) and interferon (IFN)-γ (recombinant mouse, expressed in *E. coli*) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

The murine macrophage/monocyte cell line RAW 264.7 cells from American Type Culture Collection (ATCC) were maintained at 37°C in humidified 5% CO_2/95% air in DMEM containing 10% (fetal bovine serum (FBS) Hyclone, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated in culture dishes at a density of 1×10^5 cells/well in 96-well plates for the nitrite assay and at a density of 1×10^7 cells/100-mm dish for the preparation of cytosolic fractions, nuclear extracts, or total RNA isolation.

Measurement of Nitrite Production Using Griess Reagent

NO production in culture supernatant was spectrophotometrically evaluated by measuring nitrite, an oxidative product of NO. Nitrite was determined with the Griess reaction (Green et al., 1982) by mixing 100 μl of the culture supernatant with 100 μl Griess reagent containing equal volumes of 1% (w/v) sulfanilamide in 5% (w/v) phosphoric acid and 0.1% (w/v) *N*-(1-naphthyl)ethylenediamine solution. Absorbance was measured in a microplate reader (EL808, Bio-Tek Instrument, Inc., USA) at 540 nm using a calibration curve with sodium nitrite standards.

Cell Viability

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Thus, the cells were seeded at 1×10^5 cells/well in 96-well plates and treated with LPS/IFN-γ and sesquiterpene lactones (10 μ*M*). After incubation for 24 h, the medium was removed by pouring off, and macrophages were incubated with MTT (0.1 mg/ml) for 4 h at 37°C. Medium was then removed, and 200 μl dimethyl sulfoxide (DMSO) was added to solubilize formazan crystals, and 25 μl of 0.1 *M* glycine buffer containing 0.1 *M* NaCl (pH 10.5) was then added. Absorbance was measured at 540 and 650 nm in a microplate reader.

Measurement of PGE_2 Production by Enzyme Immunoassay

RAW 264.7 cells were plated in a 100-mm dish at a density of 5×10^6 cells/well in 10 ml DMEM and treated with 500 μ*M* aspirin for COX-1 inactivation.

After 2 h of incubation, the cells were washed twice with phosphate-buffered saline (PBS) and the culture media were changed with fresh DMEM containing 5% FBS. The cells were stimulated by the addition of LPS (10 ng/ml) and IFN-γ (10 U/ml) with or without test compounds and incubated for 18 h at 37°C. The culture media were used immediately for PGE_2 determination after appropriate dilution or stored directly at −70°C until PGE_2 measurement. Intracellular cytosol was acquired according to the manufacturer's instruction. Briefly, after decanting or aspirating culture media, cells were washed twice with PBS. The concentrations of PGE_2 in the culture supernatant were measured by a commercial competitive enzyme immunoassay (EIA, Amersham-Pharmacia, USA) according to the manufacturer's instruction.

Western Blot Analysis of iNOS and COX-2 Protein

Cell lysates were boiled in Laemmli buffer for 3 min. Cell lysates, containing 30 μg total protein, were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) on 10% or 15% slab gels, and proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked for 30 min in PBS containing 0.5% Tween 20 and 5% (w/v) dry skim milk powder and incubated overnight with primary antibody. The membranes were then washed with PBS containing 0.5% Tween 20 and incubated for 2 h with the secondary antibodies conjugated to alkaline phosphatase. Bound antibodies were detected with the enhanced amplified alkaline phosphatase immunoblot system (BIO-RAD).

mRNA Analysis

Total cellular RNA was extracted from RAW 264.7 cells using Trizol reagent according to the manufacturer's instruction. mRNA levels of iNOS and COX-2 in LPS/IFN-γ-stimulated RAW 264.7 macrophages were analyzed using reverse-transcription polymerase chain reaction (RT-PCR) and Northern blot. RT-PCR was carried out using the GeneAmp RNA PCR kit (Perkin-Elmer, USA) according to the manufacturer's instructions, and Northern blot analysis was carried out as described previously (Kim et al., 2001) using a ^{32}P-labeled (Amersham) cDNA probe and 20 μg total RNA per lane. The sense and antisense primers for iNOS were 5′-GTGTTCCACCAGGAGATGTTG-3′ and 5′-CTCCTGCCCACTGAGTTCGTC-3′; for COX-2 they were 5′-ACTTGCTCACTTTGTTGAGTCATTC-3′ and 5′-TTTGATTAGTACTGTAGGGTTAATG-3′; and for GAPDH mRNA expression they were 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and 5′-TCCTTGGAGCCATGTAGGCCAT-3′, respectively.

Electrophoretic Mobility Shift Assay (EMSA) of NF-κB

Nuclear proteins were extracted by modified method of Andrews and Faller (1991). All the procedures for nuclear protein extraction were performed at 0 to 4°C with ice-cold reagents. Scraped and pelleted cells were resuspended in 1 ml ice-cold lysis buffer and incubated for 15 min on ice with occasional vortexing. After centrifugation and washing of nuclei pellet, 30–50 μl

ice-cold hypertonic extraction buffer was added and incubated at 4°C for 40 min with constant shaking. Nuclear extracts were isolated by centrifugation at 20,000 × g for 30 min and protein content in aliquots were determined by Bradford (1976) assay. Nuclear extracts were stored at −70°C until use for EMSA. The oligonucleotide probe used for EMSA contained the NF-κB consensus sequence. Double-stranded NF-κB consensus sequence was obtained from Bioneer Corp. (Chungbuk, Korea) and used for radioactive labeling after annealing. The sequences of probes used in this work are shown as follows (binding site underlined).

NF-κBU 5′-AGC-TTG-GGG-ACT-TTC-C-3′
NF-κBL 3′-C-CCC-TGA-AAG-GTC-GGC-5′

One nanomole of each oligonucleotide was annealed by heating at 95°C for 5 min and cooling to 30°C, and diluted to 1.75 pmol/μl. Oligonucleotide probe was labeled with α-[^{32}P]dATP using Klenow fragment (BM). The total volume of labeling mixture was 25 μl and the composition of labeling mixture was as follows: 7 pmol oligomer (DNA probe), 0.4 m*M* dNTPs (without dATP), labeling buffer, 4 μl α-[^{32}P]dATP (> 3000 Ci/mmol, 10 Ci/μl), and 1 μl Klenow fragment (1 U/μl). The labeling reaction was performed for 40 min at 37°C and the labeled probes were purified by Sephadex G-25 spin-column chromatography.

Binding reactions were performed at room temperature for 30 min with 5–10 μg nuclear protein in 20 μl binding buffer containing 1 μg Poly[dI–dC·dI–dC] and 100,000 cpm ^{32}P-labeled probe. The specificity of the binding reaction was confirmed by competition assay with a 100- to 200-fold molar excess of unlabeled oligonucleotide probe. DNA–protein complex was separated from unbound probe on native 6% polyacrylamide gels in 0.25 × TBE running buffer. After electrophoresis, the gel was vacuum-dried and analyzed by a Fuji BAS 2000 radioactivity detection system.

RESULTS

Zaluzanin-C and Estafiatone Inhibited Nitrite Production in a Concentration-Dependent Manner

The inhibitory effect of zaluzanin-C and estafiatone on LPS/IFN-γ-induced NO production was examined. RAW 264.7 cells were treated with zaluzanin-C and estafiatone in a range of concentrations (0.1–10 μ*M*) and then stimulated with LPS (10 ng/ml)/IFN-γ (10 U/ml) for 24 h. As shown in Figure 2, zaluzanin-C and estafiatone inhibited nitrite production in a concentration-dependent manner. The approximate IC50 value of zaluzanin-C and estafiatone is 6.61 and 3.80 μ*M*, respectively. Cell viability was also determined by MTT assay. In the presence of zaluzanin-C and estafiatone at concentration up to 10 μ*M*, cell viabilities were not different from those of

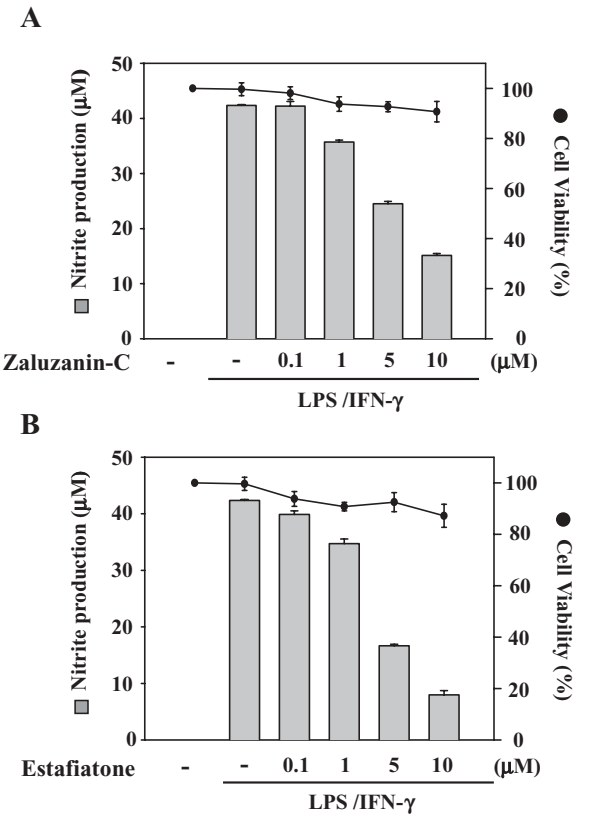

FIGURE 2. Effects of zaluzanin-C and estafiatone on the LPS (10 ng/ml)/IFN-γ (10 U/ml)-induced NO production and cell viability. RAW 264.7 cells (1×10^5 cell) were incubated at 37°C for 24 h in 200 μl of the medium containing the indicated concentrations of zaluzanin-C and estafiatone. NO production and cell viability were measured by Griess reaction and MTT assay, respectively. Total amounts of NO in the conditioned medium were determined. ■, Nitrite production; ●, cell viability.

LPS/IFN-γ-stimulated cells. Thus, these inhibitory effects were not the result of cytotoxicity.

Zaluzanin-C and Estafiatone Decreased LPS/IFN-γ-Induced PGE_2 Production

PGE_2 in the supernatant of cultured RAW 264.7 cells stimulated with LPS (10 ng/ml)/IFN-γ (10 units/ml) for 18 h in the presence of test compounds was measured. As shown in Figure 3, while unstimulated cells synthesized 0.2–0.4 ng of PGE_2, LPS/IFN-γ-stimulated cells showed about 4.3-fold increased PGE_2 synthesis. Since the cells were pretreated with acetylsalicylic acid to irreversibly inactivate cyclooxygenase by acetylation, this increased PGE_2 production indicates enhanced de novo synthesis of COX-2 enzyme (Figure 4A). However,

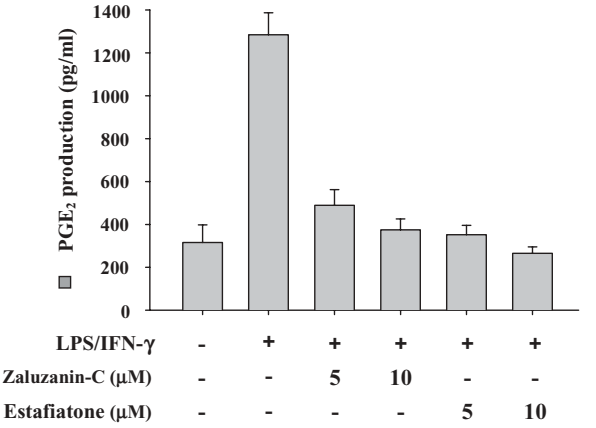

FIGURE 3. Effects of zaluzanin-C and estafiatone on the LPS (10 ng/ml)/IFN-γ (10 U/ml)-induced prostaglandin E_2 production. RAW 264.7 cells (5×10^5 cell) were incubated at 37°C for 18 h in 1 ml of the medium containing the indicated concentrations of zaluzanin-C and estafiatone. Total amounts of prostaglandin E_2 (PGE_2) in the conditioned medium were determined.

zaluzanin-C and estafiatone inhibited PGE_2 synthesis in LPS/IFN-γ-stimulated RAW 264.7 cells in a concentration-dependent manner (Figure 3).

Zaluzanin-C and Estafiatone Inhibited iNOS and COX-2 Expression at Transcriptional Level

Since both zaluzanin-C and estafiatone inhibited NO and PGE_2 production in RAW 264.7 macrophages stimulated with LPS/IFN-γ, the expression level of iNOS and COX-2 was studied in order to elucidate the inhibition mechanism exerted by these two compounds (Figure 4). Although hardly detected in unstimulated RAW 264.7 cells by Western blot analysis, iNOS and COX-2 protein was sufficiently expressed after stimulation with LPS (10 ng/ml)/IFN-γ (10 U/ml) for 20 h, and the presence of zaluzanin-C and estafiatone in LPS/IFN-γ stimulated cell cultures markedly decreased iNOS and COX-2 protein expressions in a concentration-dependent manner (Figure 4A). RT-PCR and Northern blot analysis were performed to identify whether these compounds inhibit iNOS and COX-2 expression at transcriptional level, and it was found that both zaluzanin-C and estafiatone decreased concentration-dependently the steady-state levels of iNOS and COX-2 mRNA (Figure 4, B and C). These results indicate that the inhibition of NO and PGE_2 production by two sesquiterpene lactones might be due to inhibition of iNOS and COX-2 expressions at transcriptional level.

NF-κB Activation Induced by LPS/IFN-γ Was Inhibited by Zaluzanin-C and Estafiatone

Since the activation of NF-κB transcription factor is critical for the expression of iNOS and COX-2 (Xie et al., 1994), the activation of NF-κB in RAW

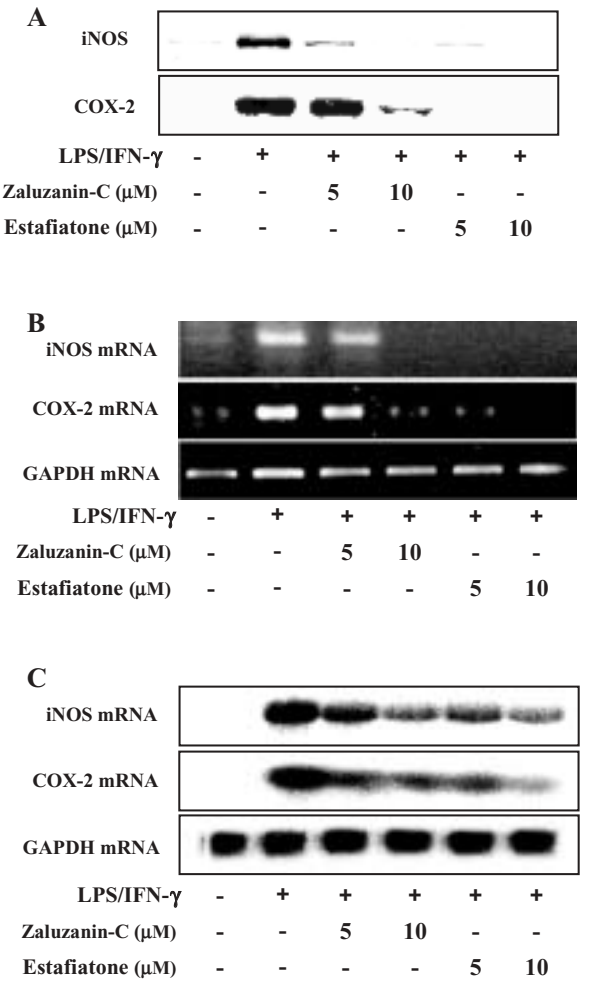

FIGURE 4. Effects of zaluzanin-C and estafiatone on the expression level of iNOS and COX-2. RAW 264.7 cells were stimulated with LPS (10 ng/ml)/IFN-γ (10 U/ml) and incubated for 20 h in the presence of test compounds. (A) Western blot analysis, (B) RT-PCR, and (C) Northern blot analysis were carried out as described in Materials and Methods.

264.7 cells was examined using EMSA to assess the effects of zaluzanin-C and estafiatone on early stages of iNOS and COX-2 gene expression (Roshak et al., 1996). Thus, the nuclear binding assay of NF-κB was carried out with nuclear extracts obtained from RAW 264.7 cells stimulated with LPS (10 ng/ml)/IFN-γ(10 U/ml) for 1 h in the presence or absence of the test compounds. Stimulation with LPS/IFN-γ markedly increased the DNA binding activity of NF-κB (Figure 5). Zaluzanin-C showed only a slight inhibition at 5 μ*M* treatment, whereas estafiatone markedly decreased DNA binding activity of NF-κB

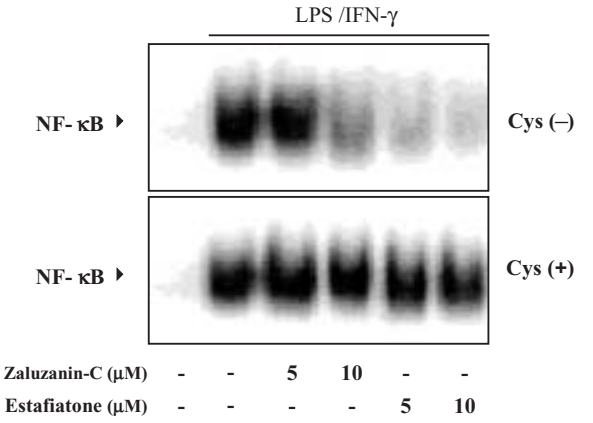

FIGURE 5. Effects of zaluzanin-C and estafiatone on the DNA binding activity of NF-κB in RAW 264.7 macrophages treated with LPS/IFN-γ. RAW 264.7 cells were stimulated with LPS (10 ng/ml)/IFN-γ (10 U/ml) for 1 h after 1 h pretreatment with zaluzanin-C and estafiatone in the absence or presence of 1 m*M* cysteine (Cys). Nuclear extracts were analyzed for DNA binding activity.

stimulated for 1 h. These results suggest that zaluzanin-C and estafiatone blocked iNOS and COX-2 expression by affecting NF-κB activation.

Exomethylene group in sesquiterpene lactones reacts with nucleophiles, especially sulfhydryl groups of some proteins such as NF-κB, by a Michael-type addition. Based on computer molecular modeling, Rüngeler et al. (1999) proposed how helenalin, a sesquiterpene lactone, may alkylate p65 subunit of NF-κB. It may cross-link two cysteine residues, Cys 38 being located within the DNA binding domain and Cys 120 being found in a proximal loop, thereby inhibiting DNA binding of NF-κB. To show that NF-κB inhibition is due to irreversible alkylation of free sulfhydryls on cysteine residues of protein, an excess of cysteine (1 m*M*) was added 1 h before LPS/IFN-γ stimulation. The addition of 1 m*M* cysteine abolished the inhibitory effect of zaluzanin-C and estafiatone on the DNA binding activity of NF-κB (Figure 5). These results indicate that inhibitory effect of zaluzanin-C and estafiatone on DNA binding activity of NF-κB might be due to either the direct modification of NF-κB or the inhibition of upstream events leading to NF-κB activation.

Zaluzanin-C and Estafiatone Inbihited IκB-α Degradation and Phosphorylation

It has been reported that IKK can phosphorylate IκB-α and IκB-β, thereby targeting them for phosphorylation and degradation through the ubiquitin–proteasome pathway (Chen et al., 1995). To determine whether zaluzanin-C and estafiatone affected phosphorylation and degradation of IκB-α, the levels of IκB-α were determined in RAW 264.7 cells stimulated with LPS (1 mg/ml)/IFN-γ (10 U/ml) for 30 min in the presence or absence of zaluzanin-C and estafiatone. Western blot analysis of cytosolic fractions

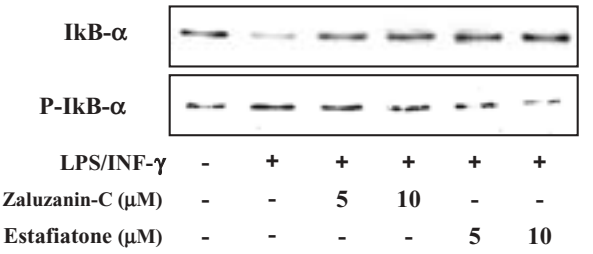

FIGURE 6. Effect of zaluzanin-C and estafiatone on the degradation and phosphorylation of IkB-α in RAW 264.7 cells. After treating with zaluzanin-C and estafiatone for 1 h, RAW 264.7 cells were stimulated with LPS (1 mg/ml)/IFN-γ (10 U/ml) for 30 min. Cellular extracts were analyzed by Western blotting with an anti-IkB-α and anti-phospho-IkB-α polyclonal antibody.

with antibodies specific for IκB-α and phospho-IκB-α showed that LPS/IFN-γ produced marked increase in the level of both IκB-α phosphorylation and degradation. However, zaluzanin-C and estafiatone blocked degradation and phosphorylation of IκB-α in a concentration-dependent manner (Figure 6).

DISCUSSION

Since increased NO and PGE_2 production is associated with acute and chronic inflammation, intracellular levels of iNOS and COX-2 play a central role in determining NO and PGE_2 production rates in activated macrophages and several other cell types. The regulation of iNOS and COX-2 has been an important target in the field of remedy of inflammation (Rao, 2000; Szabo & Thiemermann, 1995; MacMicking et al., 1997). Therefore, two sesquiterpene lactones, zaluzanin-C and estafiatone, isolated from *A. acerifolia* were investigated for their potential therapeutic values by determining their effect on iNOS and COX-2.

A. acerifolia has been used in Korean medicine as a remedy for treating pain and inflammation. The active principle in *A. acerifolia* that exhibits these beneficial effects, however, has not been determined. Results of this study indicated that the sesquiterpene lactones zaluzanin-C and estafiatone inhibited not only the LPS/IFNγ-induced nitrite and PGE_2 production (Figures 2 and 3) but also the induced iNOS and COX-2 gene expression (Figure 4) in macrophages without appreciable cytotoxic effects. These results suggest that the inhibitory effects induced by these compounds were mediated via a reduction of iNOS and COX-2 mRNA expression. Therefore, data demonstrated that iNOS and COX-2 gene expression in RAW 264.7 macrophages were suppressed by zaluzanin-C and estafiatone, and the inhibition of iNOS and COX-2 gene expression was evident by reduction of inducible NO and PGE_2.

It is known that inactive NF-κB normally binds to IκB in the cytosol, and that NF-κB is activated by various factors such as cell damaging signals, bacterial infection, proinflammatory cytokines, and reactive oxygen intermediates, all of which increase protein phosphorylation and the proteolysis of IκB protein (Ghosh et al., 1998). The free and activated NF-κB is in turn translocated into the nucleus to bind relevant DNA sites on promoter region of its target genes, including various inflammatory cytokine genes such as IL-1, IL-2, IL-6, IL-8, and TNF-α, as well as genes encoding cell adhesion molecules, immunoreceptors, hematopoietic growth factors, and growth factor receptors (Baeuerle & Henkel, 1994; Hong et al., 2002). NF-κB is also a crucial transcription factor for mRNA expression of iNOS (Xie et al., 1993, 1994) and COX-2 (Lee et al., 2004). The central role of NF-κB in mediating inflammatory processes is evident from both the importance of its target genes and from the phenotypes of mice lacking the NF-κB subunit (Traenckner et al., 1994). Therefore, compounds inhibiting NF-κB are potentially of great interest for developing therapeutic agents for the treatment of acute and chronic inflammation.

It has been reported that the sesquiterpene lactones parthenolide and isohelenin, isolated from the extracts of Mexican Indian medicinal plants, inhibit iNOS gene expression by stabilizing the association between IκB-α and NF-κB (Wong & Menendez, 1999). Therefore, to identify the effect of these compounds isolated from *A. acerifolia* on NF-κB, EMSA was carried out. When treated with zaluzanin-C and estafiatone subsequent to stimulation with LPS/IFN-γ, the DNA binding activity of NF-κB in RAW 264.7 cells was completely diminished (Figure 5). The inhibition of DNA binding activity of NF-κB by zaluzanin-C and estafiatone might be due to irreversible alkylation of free sulfhydryls on cysteine residues because the addition of 1 m*M* cysteine abolished the inhibitory effect of zaluzanin-C and estafiatone (Figure 5). Furthermore, zaluzanin-C and estafiatone prevented the phosphorylation and degradation of IκB-α from the cytosolic fraction, inhibiting the transcription of genes dependent on NF-κB activity (Figure 6). These data showed that zaluzanin-C and estafiatone directly inhibited not only the DNA binding activity of NF-κB but also IκB degradation by treatment with LPS/IFN-γ. However, because zaluzanin-C and estafiatone may inhibit a step leading to the activation of the IκB kinase or affecting the functioning of 26 S proteasome, further studies are required.

In conclusion, data suggest that zaluzanin-C and estafiatone from *A. acerifolia* suppress NO and PGE_2 synthesis in RAW 264.7 macrophages through the inhibition of iNOS and COX-2 gene expression. The inhibition of iNOS and COX-2 gene expression is, in large part, associated with the inhibition of nuclear translocation of NF-κB through suppression of IκB phosphorylation and degradation as well as direct modification of active NF-κB. Furthermore, the ability to block iNOS and COX-2 gene expression may account, in part, for the anti-inflammatory properties of zaluzanin-C and estafiatone.

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