Aqueous Extract of *Oldenlandia diffusa* Suppresses LPS-Induced iNOS, COX-2 and TNF-α Expression in RAW 264.7 Cells via the NF-κB Activity

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Abstract

**Purpose:** To elucidate the anti-inflammatory mechanisms of aqueous extract of *Oldenlandia diffusa* (AEOD) in LPS-stimulated RAW 264.7 cells.

**Methods:** We evaluated the mRNA and protein expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and tumor necrosis factor (TNF-α) using RT-PCR and Western blot analyses. Expressions of IκBα, phospho-κBα and p65 were analyzed by Western blot analysis. The level of nitric oxide (NO) production was analyzed using Griess reaction. The release of prostaglandin E₂ (PGE₂) and tumor necrosis factor (TNF-α) was determined using sandwich ELISA.

**Results:** AEOD significantly suppressed nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells without direct cytotoxicity. AEOD decreased the production of prostaglandin E₂ (PGE₂) and TNF-α in LPS-stimulated RAW 264.7 cells. LPS-induced mRNA and protein expression of iNOS, COX-2 and TNF-α were attenuated by treatment with AEOD. These data imply that AEOD tightly regulates the expression of these inflammatory mediators at the transcriptional level. Therefore, we determined the effects of AEOD on nuclear factor-κB (NF-κB) activity, which has been considered to be a potential transcriptional factor for regulating the expression of iNOS, COX-2 and TNF-α. As expected, AEOD suppressed the LPS-induced degradation and phosphorylation of IκBα and sustained the expression of p65 in the cytosol. Furthermore, AEOD substantially inhibited the LPS-induced DNA binding activity of NF-κB. These data show that AEOD may control NO, PGE₂ and TNF-α production via the suppression of NF-κB activity.

**Conclusion:** Our results suggest that AEOD has a high potential activity for regulating LPS-induced inflammation.

**Keywords:** Oldenlandia diffusa, NO, iNOS, COX-2, PGE₂, TNF-α, NF-κB

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INTRODUCTION

Activated macrophages provide an immediate defense against extracellular foreign agents and are important in maintaining a balance in the immune system. They also play a crucial role in inflammatory processes such as lipopolysaccharide (LPS)-induced shock, asthma, inflammatory bowel disease, and atherosclerosis [1]. In the inflammatory process, activated macrophages lead to the production of different kinds of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, in addition to other inflammatory mediators such as nitric oxide (NO) and prostaglandin E₂ (PGE₂) [2]. Excessive production of these cytokines and inflammatory mediators has been known to cause severe tissue damage, septic shock, atherosclerosis and systemic inflammatory response syndrome [3]. Therefore, many researchers have attempted to find naturally occurring products to suppress LPS-induced inflammation through the suppression of inflammatory mediators such as NO, PGE₂ and TNF-α.

Nuclear factor (NF)-κB has been associated with inflammation, tumor cell proliferation, invasion, angiogenesis, and metastasis through its regulation of various gene products [4]. Therefore, the regulation of NF-κB activation can potentially suppress inflammation and tumor cell proliferation. NF-κB is heterodimer consisting of p65 and p50 proteins; it is located in the cytosol and is complexed with the inhibitory protein IκBα [5]. A variety of extracellular signals, including LPS and growth factors can activate the enzyme inhibitory κB (IκB) kinase, which results in ubiquitination and degradation of IκBα by the proteasome, and the eventual dissociation of IκBα from NF-κB [6]. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA. Ultimately, NF-κB promotes the expression of inflammation-related gene products such as iNOS, COX-2 and TNF-α [7]. Therefore, modulation of NF-κB activity is a good strategy for therapy against inflammatory diseases.

*Oldenlandia diffusa* is a well-known medicinal plant used worldwide. Aqueous extract of *O. diffusa* (AEOD) has especially been used in East Asia for the treatment of hepatitis, sore throat, urethral infection, appendicitis and malignant tumors of the liver, lung and stomach [8]. It is also well known that AEOD potentially inhibits the growth of cancer cells or mutagenesis [9]. Oleanoic and ursolic acids - chemical constituents isolated from this plant species - are as effective as antibiotics, and anti-cancer, liver protection and transaminase-degrading agents [10]. Nevertheless, little is known about the molecular mechanisms by which AEOD regulates LPS-induced inflammation.

In this study, we investigated the effects of AEOD on the expression of NO, PGE₂, and TNF-α in LPS-stimulated RAW 264.7 macrophage cells. We found that AEOD downregulates the expression of iNOS, COX-2 and TNF-α mRNA and attenuates the corresponding mediators in vitro in RAW 264.7 macrophage cells. We also found that AEOD inhibits LPS-induced NF-κB activation by suppressing the phosphorylation and degradation of IκBα.

EXPERIMENTAL

Preparation of AEOD

*O. diffusa* was purchased from a local Oriental herb store, Kwang Myoung Dang (Busan, Republic of Korea) in February 2005. *O. diffusa* (stock no. 200505) was identified and authenticated by Professor WS Ko (College of Oriental Medicine, Dongeui University, Busan, Republic of Korea). A voucher specimen was deposited at the Department of Oriental Medicine, Dongeui University. *O. diffusa* (100 g) was extracted with distilled water at 100 °C for 2 h, the aqueous mixture filtered through a 0.45 μm filter (Millipore, Bedford, USA) and the filtrate freeze-dried (yield, approximately 5.2 g) and kept at 4 °C. The dried filtrate was dissolved...
in phosphate buffered saline (PBS) and filtered through 0.22 µm filter before use.

Reagents

Lipopolysaccharide (LPS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, USA). Antibodies against iNOS, COX-2, p65, IκBα and phospho (p)-IκBα were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). The antibody against β-actin was from Sigma. Peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from KOMA Biotechnology (Seoul, Republic of Korea). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, Republic of Korea). Other chemicals used were purchased from Sigma.

Cell culture and viability

RAW 264.7 murine macrophage cell line was kindly provided by SJ Jeong (Kyung Hee University, Seoul, Republic of Korea) and cultured at 37 °C in 5 % CO₂ in DMEM medium supplemented with 10 % FBS and antibiotics. For the analysis of cell viability, the cells (1 × 10⁵ cells/ml) were incubated with the indicated concentrations of AEOD for 1 h before the treatment with LPS (1.0 µg/ml) for 24 h. Cell viability was determined by MTT assay.

Isolation of total RNA and RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instruction. One microgram RNA was reverse-transcribed using MMLV reverse transcriptase (Promega, Madison, USA). cDNA was amplified by PCR using specific primer, iNOS (forward 5’-ctt gtt ggt ggg aay ggg tc-3’ and reverse 5’-ttt ggt gtc acg cac gat tt-3’). The following PCR conditions were applied: COX-2 and iNOS, 25 cycles of denaturation at 95 °C for 45 s, annealing at 59 °C for 45 s, and extended at 72 °C for 1 min. β-Actin was used as an internal control to evaluate relative expression of COX-2, iNOS and TNF-α.

Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction kit (iNTRON Biotechnology; Sungnam, Republic of Korea). Briefly, after treatment with the indicated concentrations of AEOD, cells were harvested, washed once with ice-cold PBS and gently lysed for 15 min in 100 µl ice-cold PRO-PREP lysis buffer. Lysates were centrifuged at 14,000 g and 4 °C for 10 min to obtain the supernatants. The supernatants were collected and protein concentrations determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, USA). The samples were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, USA). Proteins were detected using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, USA).

NO assay

RAW 264.7 cells (2 × 10⁵ cells/ml) were plated onto 24-well plates and pretreated with the indicated concentrations of AEOD 1 h prior to stimulation with 1.0 µg/ml of LPS for 24 h. Supernatants were collected and assayed for NO production using Griess reagent. Briefly, the samples were mixed with equal volume of Griess reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphthylethenediamine dihydrochloride) and then incubated at room temperature for 10 min. The absorbance was measured at 540 nm on a microplate reader (Thermo Scientific; Waltham, USA).

Trop J Pharm Res, August 2011;10 (4):405
Electron Corporation, Marietta, USA). Nitrite concentration was determined using a dilution of sodium nitrite as a standard.

**Measurement of TNF-α and PGE₂**

The expression levels of PGE₂ and TNF-α were measured by an enzyme immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, USA) according to the manufacturer’s instructions. Briefly, RAW 264.7 cells (2 × 10⁵ cells/ml) were plated in 24-well plates and pretreated with the indicated concentrations of AEOD for 1 h prior to stimulation with 1.0 µg/ml of LPS for 24 h. One hundred microliters of culture-medium supernatant was collected for determination of PGE₂ and TNF-α concentration by ELISA.

**Electrophoretic mobility assay (EMSA)**

DNA-protein binding assays were carried out with nuclear extract. Synthetic complementary NF-κB (5’-AGT TGA GGG GAC TTT CCC AGG C-3’) binding oligonucleotides (Santa Cruz Biotechnology) were 3’-biotinylated using the biotin 3’-end DNA labeling kit (Pierce) according to the manufacturer’s instructions, and annealed for 30 min at room temperature. Assays were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5 × Tris borate/EDTA before being transferred onto a positively charged nylon membrane (HybondTM-N+) in 0.5 × Tris borate/EDTA at 100 V for 30 min. The transferred DNAs were cross-linked to the membrane at 120 mJ/cm². Horseradish peroxidase-conjugated streptavidin was used according to the manufacturer’s instructions to detect the transferred DNA.

**Statistical analysis**

All data were derived from at least three independent experiments. Statistical analyses were conducted using SigmaPlot software (version 11.0) and values presented as mean ± standard deviation (SD). Significant differences (p < 0.01) between the groups were determined using the unpaired Student’s t-test.

**RESULTS**

**Effect of AEOD on the viability of RAW 264.7 macrophage cells**

To assess whether AEOD influences cell viability in RAW 264.7 cells, an MTT assay was performed at 24 h after treatment with the indicated concentrations of AEOD in the presence or absence of LPS. We did not observe any effects on cell viability in RAW 264.7 cells by treatment of up to 2.0 mg/ml of AEOD alone (Fig. 1). Additionally, in the presence of LPS (1.0 µg/ml), AEOD did not affect the viability of the cells. Cytotoxicity appeared at concentrations of over 2.5 mg/ml of AEOD (data not shown). Therefore, the concentration of AEOD was applied within this range in the remaining experiments.

**Effect of AEOD on LPS-induced NO and PGE₂ production**

To investigate the anti-inflammatory effects of AEOD, we examined the effects of AEOD on NO and PGE₂ production in LPS-stimulated RAW 264.7 cells. The production of NO and PGE₂ was analyzed using the Griess reaction assay and ELISA, respectively. The level of NO was reflected by the accumulation of nitrite in the cell culture medium. Stimulation of cells with LPS resulted in a significant increase in NO production. PGE₂ production was also increased by LPS stimulation, and AEOD treatment significantly reduced this production.

**Figure 1:** Effect of AEOD on the viability of RAW 264.7 macrophage cells (n = 3).
increase in nitrite production (17.6 ± 0.1 µM) compared to the untreated control (6.8 ± 0.3 µM; Fig. 2A). However, pre-treatment with 2.0 mg/ml AEOD significantly reversed LPS-stimulated nitrite production to levels near the control (8.2 ± 0.5 µM). Consistent with the suppression of nitrite production, the exposure of LPS induced an increase in PGE₂ release from 145 ± 54 pg/ml to 3420 ± 112 pg/ml. LPS-induced PGE₂ release also decreased in the presence of AEOD (1623 ± 56 pg/ml), but levels did not reach those of the control (Fig. 2B). Taken together, these data indicate that AEOD significantly suppresses the release of NO and PGE₂ in LPS-stimulated RAW 264.7 cells.

**Figure 2:** Effects of AEOD on LPS-induced nitrite (A) and PGE₂ (B) release in RAW 264.7 cells (n = 3); significantly different from the value in cells treated with LPS alone (p < 0.01)

**Effect of AEOD on LPS-induced iNOS and COX-2 protein and mRNA**

Both RT-PCR and Western blot analyses were conducted to determine the inhibitory effects of AEOD on the expression of iNOS and COX-2 proteins and mRNA. Western blot analysis showed that treatment with LPS significantly increased the expression of iNOS and COX-2 proteins at 24 h. However, the expression was dose-dependently attenuated by pretreatment with AEOD for 1 h (Fig. 3A). To assess whether the downregulation of iNOS and COX-2 is regulated at the transcriptional level, we performed RT-PCR in a simultaneous condition. RT-PCR analysis also showed that the decreasing patterns of iNOS and COX-2 mRNA expression were similar to those of their protein levels (Fig. 3B). These data indicate that AEOD suppresses the upregulation of LPS-stimulated iNOS and COX-2 expression at the transcriptional levels.

**Figure 3:** Effect of AEOD on LPS-induced iNOS and COX-2 protein (A) and mRNA (B) expression in RAW 264.7 cells (n = 3).

**Effect of AEOD on LPS-induced TNF-α production and mRNA expression**

Next, we attempted to test the potential effects of AEOD on TNF-α mRNA release and expression in LPS-stimulated RAW 264.7 cells. TNF-α was weakly expressed in untreated controls (632 ± 23 pg/ml). However, LPS stimulation remarkably increased TNF-α release at 24 h (5423 ± 36 pg/ml; Fig. 4A). Pre-treatment with AEOD reversed LPS-induced TNF-α release to a level slightly higher than the control (1523 ±
17 pg/ml), though AEOD itself did not alter the release in the absence of LPS. To review whether the downregulation of AEOD-induced TNF-α release was due to the regulation of the TNF-α gene in LPS-stimulated RAW 264.7 cells, we performed a semi-quantitative RT-PCR analysis 6 h after LPS treatment. AEOD reduced the expression of TNF-α mRNA in LPS-stimulated RAW 264.7 cells in a dose-dependent manner (Fig. 4B). These data indicate that AEOD regulates LPS-stimulated TNF-α release at the transcriptional level.

**Figure 4:** Effect of AEOD on LPS-induced TNF-α expression in RAW 264.7 cells (n = 3).

**Inhibitory effect of AEOD in LPS-induced NF-κB activity**

Since it is well known that NF-κB activation by LPS induces the expression pro-inflammatory mediators such as iNOS, COX-2 and TNF-α, we used Western blot analysis and an EMSA to investigate how AEOD regulates the specific DNA binding activity of NF-κB. LPS significantly increased the phosphorylation and degradation of IκBα and reduced the expression of p65 in the cytosol. However, treatment with AEOD alone sustained the same levels of IκBα and p65 compared to the control group (Fig. 5A). In addition, AEOD attenuated LPS-induced NF-κB activation. In a parallel experiment, EMSA was conducted to determine whether AEOD inhibits the DNA-binding activity of NF-κB. LPS caused a remarkable increase in binding complexes between NF-κB and specific-binding DNA; it was found that pre-treatment with AEOD significantly reduced LPS-induced NF-κB activity (Fig. 5B). These data indicate that AEOD results in reduced NF-κB activity in LPS-stimulated RAW 264.7 cells by suppressing the phosphorylation of IκBα.

**Figure 5:** Effect of AEOD on IκBα degradation, IκBα serine 32 phosphorylation and p65 protein expression (A) and NF-κB DNA binding activity (B) in LPS-stimulated RAW 264.7 cells (n = 3).

**DISCUSSION**

It is well-known that in macrophages, LPS induces the expression of iNOS, COX-2, and TNF-α, which play a key role in the pathogenesis of inflammatory conditions [1,2]. Recently, many researchers have attempted to find naturally occurring products to downregulate these inflammatory mediators, because this is thought to be a good strategy for curing LPS-induced inflammation. *O. diffusa* is a commonly used medicinal plant for treating cancer. A recent report showed that AEOD exhibits cancer cell-specific cytotoxicity in various types of
human cancer cells, both in vivo and in vitro [11]. Furthermore, a recent study has shown that major components of O. diffusa, such as oleanolic and ursolic acids, also exhibit growth inhibition against Ras-transformed fibroblasts as well as anti-arthritic effects in acute and chronic arthritis models [12]. Nevertheless, the molecular mechanisms underlying the effects of AEOD on LPS-stimulated inflammation are not well known. In the present study, we investigated the anti-inflammatory effects of AEOD in vitro in LPS-stimulated RAW 264.7 murine macrophage cells. The results showed that non-toxic concentrations of AEOD significantly suppressed the release of NO and PGE$_2$ in LPS-stimulated RAW 264.7 cells; its effects are accompanied by a decrease in the expression of iNOS and COX-2 mRNA. In addition, AEOD reduced the release and expression of the proinflammatory cytokine TNF-α. Further experiments showed that AEOD attenuates LPS-induced NF-κB activity by downregulating the phosphorylation and degradation of IkBα (fig 6).

**Figure 6:** A schematic diagram of the anti-inflammatory effects of AEOD in LPS-stimulated RAW 264.7 cells (Note: AEOD represses the release of inflammatory mediators such as NO, PGE$_2$ and TNF-α via the suppression of NF-κB activity).

NO generated from the amino acid, L-arginine, by NOS, is involved in regulating physiological and pathological processes [13]. There are 3 known isoforms of NOS: the first 2 are constitutive and the third is inducible [14]. NO production by iNOS especially promotes anti-bacterial and anti-cancer responses. However, overproduction of NO has also been associated with the initiation and maintenance of inflammation [13]. Therefore, many researchers are developing agents to regulate the expression of NO via iNOS [15,16]. In the present study, we report that AEOD inhibits the production of NO via the suppression of iNOS expression.

PGE$_2$ is another key inflammatory mediator involved in inflammatory responses [17]. Two isoforms of COX enzymes exist: COX-1 and COX-2. Also known as COX-2, prostaglandin-endoperoxide synthase 2 is an enzyme encoded by the PTGS2 gene in humans; the activation of this gene is responsible for various inflammatory diseases. Aberrant upregulation of PGE$_2$ has often been observed in inflammatory and malignant tissues [18]. There is accumulating evidence that confirms COX-2 as a potential therapeutic target for the treatment of inflammation and cancer [18]. The present study revealed that AEOD down-regulates PGE$_2$ production via the suppression of COX-2 expression. Kang et al. [19] reported that oral feeding of ursolic acid one of the major components of AEOD, suppressed zymosan-induced PGE$_2$ production in air pouch exudates and adjuvant-induced chronic arthritis, without significant gastric lesions; this is consistent with our data. Taken together, these data indicate that AEOD is a potential therapeutic candidate for alleviating LPS-stimulated up-regulation of PGE$_2$ and COX-2.

TNF-α is a cytokine involved in the inflammatory and destructive process common to several human inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, and insulin-dependent diabetes mellitus [20]. Although each inflammatory disease exhibits different symptoms, the pathogenesis of these diseases is similar in terms of high
expression level of TNF-α in damaged tissues. In many cells, overproduction of TNF-α switches on the signaling pathways that change the cell functions of LPS-stimulated macrophages [21]. Recently, anti-TNF-α therapy has been broadly introduced to treat various inflammatory diseases [20]. Although cytokine therapy is an effective strategy, the demand for combination therapy is increasing because of its efficacy and side effects of TNF-α alone [21,22]. Therefore, naturally occurring products with no side effects in normal cells have been considered to be potential therapeutic agents. In this study, we attempted to demonstrate that AEOD attenuates LPS-induced TNF-α production in RAW 264.7 cells. Our results showed that AEOD significantly reduced LPS-induced TNF-α production by inhibiting TNF-α mRNA expression.

NF-κB is located in the cytoplasm as an inactivated dimer composed of p65 and p50 subunits. In response to pro-inflammatory stimuli, IκBα is phosphorylated and degraded, and NF-κB is released and translocated to the nucleus [4,5]. Since the expression of many inflammatory genes, including iNOS, COX-2, and TNF-α, are known to be modulated by NF-κB binding to their specific promoter regions [23], we tested the expression of p65 and IκB as well as the specific DNA-binding activity of NF-κB. In addition, AEOD decreased the LPS-induced DNA-binding activity of NF-κB. Taken together, these results suggest that AEOD suppressed LPS-induced NF-κB activity by suppressing the degradation and phosphorylation of IκB.

CONCLUSION

This study confirmed that AEOD has anti-inflammatory activity, which works by regulating the production of NO, PGE₂, and TNF-α via the suppression of LPS-induced activation of NF-κB.

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DISCLOSURE

No competing interests exist.

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