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Anti-inflammatory Effect of Achyranthoside E Dimethyl Ester in LPS-stimulated RAW 264.7 Cells

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Achyranthoside E dimethyl ester (AEDE) is an oleanolic acid glycoside from *Achyranthes japonica*. In this study, we investigated the effects of AEDE on nitric oxide (NO) production and underlying molecular mechanisms in lipopolysaccharide (LPS)-stimulated macrophages. AEDE inhibited LPS-induced NO secretion as well as inducible NO synthase (iNOS) expression, without affecting cell viability. Further study demonstrated that AEDE induced heme oxygenase-1 (HO-1) gene expression. In addition, the inhibitory effects of AEDE on iNOS expression were abrogated by small interfering RNA-mediated knock-down of HO-1. Moreover, AEDE induced nuclear translocation of nuclear factor E2-related factor 2 (Nrf2), a transcription factor that regulates HO-1 expression. AEDE-induced expression of HO-1 was inhibited by inhibitors of phosphatidylinositol 3-kinase (PI-3K) and extracellular signal regulated kinase (ERK1/2). AEDE phosphorylated Akt and ERK1/2 as well. Therefore, these results suggest that AEDE suppresses the production of pro-inflammatory mediator such as NO by inducing HO-1 expression via PI-3K/Akt/ERK-Nrf2 signaling. These findings provide the scientific rationale for anti-inflammatory therapeutic use of AEDE.

Key words : Achyranthoside E dimethyl ester, heme oxygenase-1, inducible nitric oxide synthase, NF-E2-related factor 2, nitric oxide

Introduction

Achyranthoside E dimethyl ester (AEDE) is an oleanolic acid glycoside from *Achyranthes japonica* and *Achyranthes bidentata* [16]. *A. japonica* and *A. bidentata* have been used in traditional medicine for the treatment of edema, arthritis, and delayed menses and as a contraceptive and abortifacient [1]. *A. japonica* has been reported to inhibit platelet aggregation [36] and have anti-fungal [14] and anti-inflammatory activities [9]. Our previous study demonstrated that *A. japonica* extract has anti-inflammatory activity [3]. *A. bidentata* has been reported to have anti-inflammatory [10, 18, 33], osteoprotective [11, 35, 37] and neuroprotective [27, 35, 38] activities. AEDE from *A. bidentata* has inhibitory activity on osteoclast formation [16]. However, little is known about the effects of AEDE on inflammation and underlying mechanisms.

Nitric oxide (NO) is a free radical with multiple effects on various organ systems. The most prominent physiological actions of NO as a biological mediator include cGMP-dependent vasodilation, neural communication, host defense, inflammation, immune suppression and blood clotting [20]. NO is produced in physiological and pathophysiological conditions by NO synthase (NOS), and inducible NOS (iNOS) is induced by inflammatory cytokines and/or bacterial lipopolysaccharide (LPS) in various cell types including macrophages. A large amount of NO, particularly synthesized by iNOS, induces an inflammatory response to inhibit the growth of invading microorganisms and tumor cells. This strong inflammatory response to foreign cells could also cause further damage for the neighboring cells and tissues of the host [19]. Therefore isozyme specific inhibitors of NOS are essential for therapeutic purposes and drugs that specifically inhibit iNOS could be useful in treating diseases mediated by NO overproduction [28].

Heme oxygenase-1 (HO-1) is an inducible enzyme that catalyzes the rate-limiting step in the oxidative degradation of cellular heme into carbon monoxide (CO), biliverdin, and free iron [25]. HO-1 and its enzymatic by-products provide

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a host defense mechanism that can protect the body against oxidative injury and also contributes to the anti-inflammatory activity of cells and tissues [22]. In activated macrophages, HO-1 expression or CO treatment inhibits the production of the pro-inflammatory cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1β [22]. Up-regulation of HO-1 expression or the administration of CO also suppresses the production of pro-inflammatory mediators such as nitric oxide (NO) and prostaglandin E2 (PGE2) [30]. Moreover, an increasing number of therapeutic agents have been reported to induce HO-1 expression and exert their anti-inflammatory effects through HO-1 induction. These studies support beneficial effects of HO-1 that may serve as a therapeutic target in inflammatory diseases.

HO-1 is primarily regulated on the transcriptional level via signaling pathways involved in survival and stress responses in different cell types. Transcription factor NF-E2-related factor 2 (Nrf2) plays a central role for inducible expression of HO-1 [29]. In basal conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) and degraded by the ubiquitindependent 26S proteasome system [12]. Under activation, Nrf2 released from Keap1 inhibition, translocates to the nucleus, heterodimerizes with Maf, and binds antioxidant response elements (AREs) located in the promoter regions of many detoxifying/antioxidant genes, including HO-1 [12, 21].

In the present study, we investigated the effects of AEDE from *A. japonica* on LPS-induced inflammatory response (NO release) in macrophages and further explored the possible mechanisms. Our results provide a molecular basis for understanding the inhibitory effects of AEDE on endotoxin-mediated inflammation.

Materials and Methods

Materials

LPS (phenol extracted *Salmonella enteritidis*), Tween-20, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies for iNOS, HO-1, Nrf-2, TATA-box binding protein (TBP), and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HO-1 siRNA was purchased from Bioneer (Daejoen, Korea). INTERFERin siRNA Transfection Reagent was purchased from Polyplus transfection (France). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corporation (San Diego, CA, USA). AEDE (Fig. 1A) isolated from the methanol-soluble fraction of the dried roots of *A. japonica* was prepared as described previously. The isolated compound was kept at 4°C in the dark until further experiments.

Cell culture

Murine macrophage RAW 264.7 cells were maintained in DMEM supplemented with glutamine (1 mM) and 10% FBS at 37°C in an atmosphere of 5% CO_2.

Measurement of nitrite concentration

NO synthesis in cell cultures was measured by a microplate assay method. To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of the Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. Nitrite concentration was determined by measuring the absorbance at 540 nm with a Vmax 96-well microplate spectrophotometer (Molecular Devices, Menlo Park, CA, USA). The sodium nitrite was used as a standard.

Cell viability assay

The cytotoxicity of AEDE was assessed using the microculture tetrazolium (MTT)-based colorimetric assay. The remaining cells after Griess reaction were used for MTT assay. MTT was added to each well (final concentration is 62.5 μg/ml). After incubation for 3 h at 37°C and 5% CO_2, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with 150 μl of DMSO. The absorbance of each well was then read at 570 nm using microplate reader.

Western blot analysis

The cells were washed with phosphate buffered saline (PBS) three times and scraped off and lysed with lysis buffer (1% Triton X-100, 1% deoxycholate). Protein concentration of lysates was determined using Bradford reagent (Bio-Rad, Hercules, CA, USA) and equal amounts of protein were separated electrophoretically using 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and then

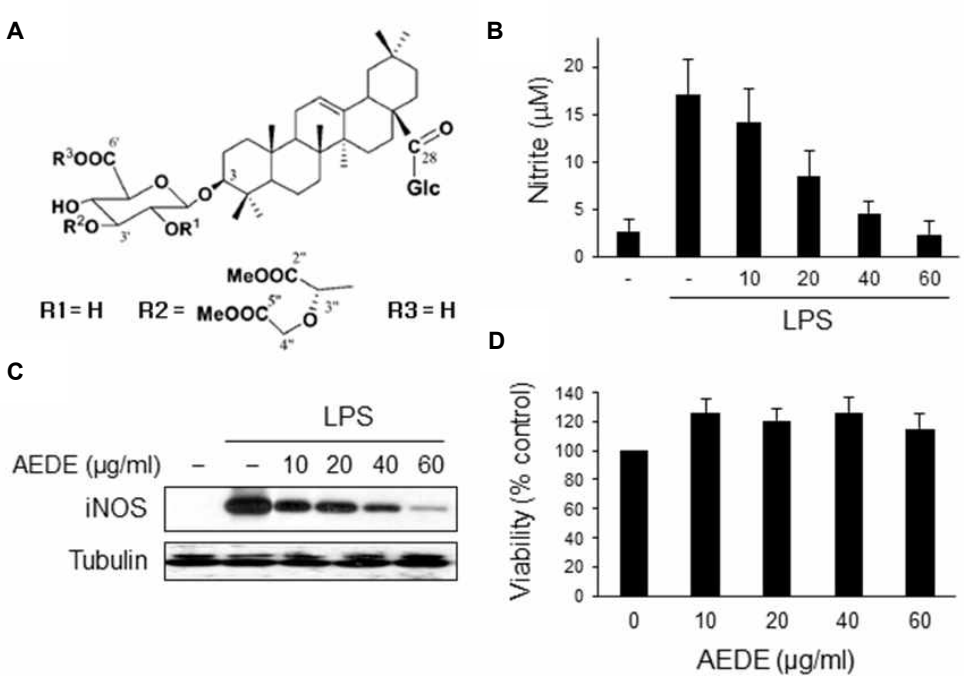

Fig. 1. Effect of AEDE on the NO secretion and iNOS expression in macrophages. (A) Chemical structure of AEDE isolated from the methanol-soluble fraction of the dried roots of *A. japonica*. (B) RAW 264.7 cells were incubated with various concentrations of AEDE for 1 h and then stimulated with LPS (0.1 μg/ml) for 20 h. The amount of nitrite released was measured by the method of Griess. (C) Cells were treated as mentioned above and equal cytosolic extracts were analyzed by Western blotting. (D) Cells were incubated with various concentrations of AEDE for 24 h and cell viability was measured by MTT assay according to *Materials and methods*. * $p<0.05$ and ** $p<0.01$ vs. LPS-treated group.

the gel was transferred to 0.45 μm nitrocellulose paper. The blot was incubated with specific antibody and then detected by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Biosciences, Piscataway, NJ, USA). TATA binding protein (TBP) was used as protein-loading controls for each lane. Quantitative image analysis was performed using image analysis software, ImageJ (http://rsb.info.nih.gov/ij) and data were presented as fold of control.

Preparation of nuclear extract

Nuclear extracts were prepared as described previously [2]. In brief, cells were washed with ice-cold PBS, centrifuged at 1,000× *g* for 5 min, resuspended in 400 μl of ice-cold hypotonic buffer (10 mM HEPES/KOH, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000× *g* for 30 sec. Pelleted nuclei were gently resuspended in 50 μl of ice-cold buffer (20 mM HEPES/KOH, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.2 mM PMSF, pH 7.9), left on ice for 30 min, vortexed, and centrifuged at 15,000× *g* for 5 min at 4°C. Aliquots of the supernatant that contained nuclear proteins were stored at -70°C.

Interference of HO-1

The siRNAs for HO-1 (GenBank accession no. NM 010442.1) were synthesized by Bioneer (Daejoen, Korea). The siRNA 1 sequence is 5′-CACCAAGGAGGUACACAUC(dTdT)-3′ (sense) and siRNA 2 sequence is 5′-CCUGAAUCGAGCAGAACCA(dTdT)-3′ (sense). Cells were transfected with HO-1 siRNA (200 nM) or negative control siRNA using INTERFERin. Then cells were incubated for 48 h until the protein expression detection.

Statistical analysis

All results were expressed as means ± SE. Each experiment was repeated at least three times. Statistical significances were compared between each treated group and analyzed by the Student's *t*-test. Data with $p<0.05$ were considered statistically significant.

Results

Effect of AEDE on NO synthesis and iNOS expression in macrophages

To investigate the anti-inflammatory effect of AEDE, we examined the effect of AEDE on NO synthesis in macrophages. RAW 264.7 cells were incubated with AEDE for 1 h and stimulated with LPS for 20 h. The amount of NO released into culture medium was measured by the method of Griess. Whereas LPS-treated cells produced a large amount of NO, AEDE suppressed NO release into culture supernatant in a dose-dependent manner (Fig. 1B). To determine whether the decreased nitric oxide synthesis is correlated with iNOS expression, we analyzed the amount of iNOS by Western blot analysis. Macrophages were treated with AEDE as mentioned above. The level of iNOS was dramatically reduced by AEDE in a dose-dependent manner (Fig. 1C). On the other hand, cell viability was not affected by AEDE as measured by MTT assay (Fig. 1D). These results suggest that AEDE inhibits NO release by suppressing iNOS expression level without affecting cell viability.

AEDE exhibits anti-inflammatory effect through the induction of HO-1

By-products of HO-1 are known to have anti-inflammatory effect [25]. Accordingly, we investigated whether AEDE exhibits its anti-inflammatory effect through the induction of HO-1 expression. We examined the HO-1-inducible activity of AEDE by Western blotting. HO-1 protein level was significantly increased by AEDE in a dose-dependent manner and reached peak at 6 h in RAW 264.7 cells (Fig. 2A, 2B). To confirm that AEDE suppresses iNOS expression through the induction of HO-1, we applied an HO-1 small interfering (si) RNA system to knock down HO-1 function. Cells were transfected with si HO-1 or si control RNA, and the effects of AEDE on iNOS expression were examined by Western blotting. As shown in Fig. 3, decreased HO-1 expression blocked AEDE-mediated suppression of LPS-stimulated iNOS expression, whereas transfection with control siRNA showed no effect. Taken together, these results indicate that HO-1 is involved in AEDE-induced inhibition of iNOS expression.

AEDE-induced HO-1 expression is mediated by Nrf2

Since the promoter region of HO-1 gene contains binding sites for transcription factor Nrf2 and the expression of HO-1 is known to be regulated by Nrf2 [29], we examined the effect of AEDE on nuclear accumulation of Nrf2 in RAW 264.7 cells. Cells were treated with AEDE, and accumulation of Nrf2 in nucleus was examined by Western blotting. Nrf2 nuclear accumulation was increased by AEDE in a dose-dependent manner and reached peak at 1 h (Fig. 4A, 4B). These results suggest that AEDE induces the expression of HO-1 through activating Nrf2.

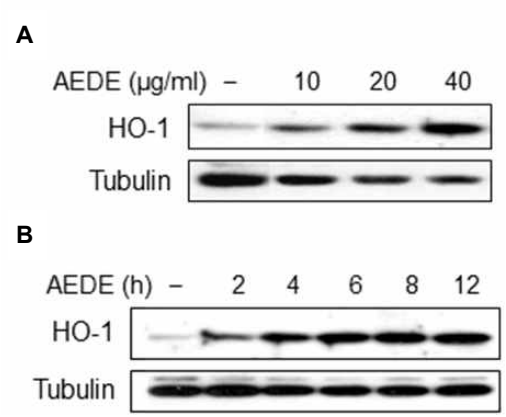

Fig. 2. Induction of HO-1 by AEDE. Cells were incubated with various concentrations of AEDE for 8 h (A), or with AEDE (40 μg/ml) for indicated times (B). Cells were harvested and equal cytosolic extracts were analyzed by Western blotting with anti-HO-1 antibody.

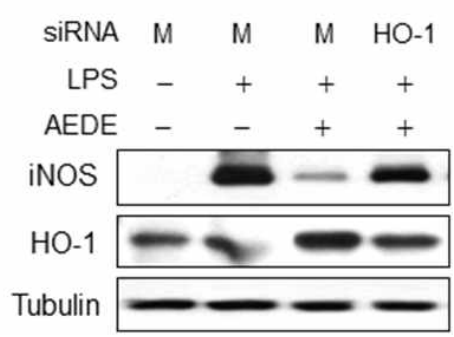

Fig. 3. Inhibitory effect of HO-1 on iNOS expression. Cells were transfected with HO-1 siRNA or control siRNA (Mock, M), after which they were pretreated with AEDE (40 μg/ml) for 1 h and stimulated by LPS (0.1 μg/ml) for 6 h. Protein levels of iNOS and HO-1 were analyzed by Western blotting.

AEDE-induced HO-1 expression is mediated by ERK1/2 pathway

We examined the signaling pathways associated with AEDE-induced HO-1 expression. RAW264.7 macrophages

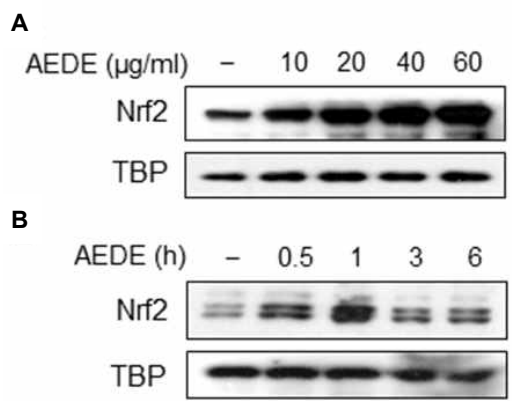

Fig. 4. Effect of AEDE on Nrf2 activation. Cells were incubated with indicated concentrations of AEDE for 1 h (A), or AEDE (40 μg/ml) for indicated times (B). Nuclear proteins were extracted and nuclear accumulation of Nrf2 was assayed by Western blotting.

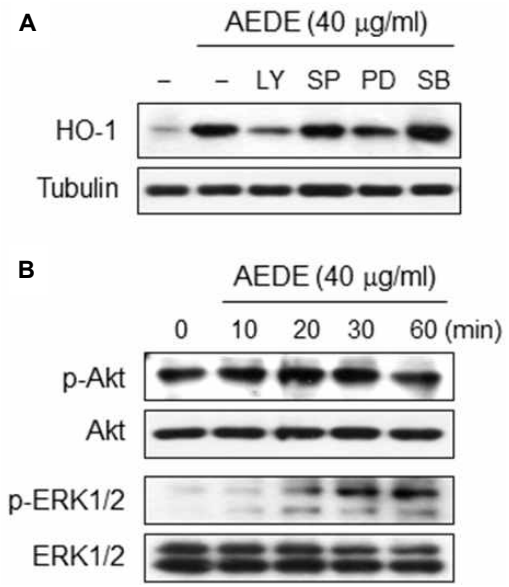

Fig. 5. Intracellular signaling pathway involved in HO-1 expression by AEDE. (A) RAW 264.7 cells were incubated with LY294002 (20 μM), SP600125 (20 μM), PD98059 (20 μM), and SB203580 (20 μM) for 1 h and then treated with AEDE (40 μg/ml) for 6 h. Equal amount of cytosolic proteins were analyzed by Western blotting with HO-1 antibody. Alpha-tubulin was used as a loading control. (B) Cells were incubated with AEDE (40 μg/ml) for indicated times. Equal amount of cytosolic proteins were analyzed by Western blotting with anti-p-Akt or -p-ERK1/2 antibodies, respectively. Akt or ERK1/2 was used as a loading control.

were exposed, separately, to one of pharmaceutical protein kinase inhibitors: LY294005 (a PI3K inhibitor), SP600125 (a JNK inhibitor), PD98059 (an ERK1/2 inhibitor) and SB203580 (a p38 MAPK inhibitor). As shown in Fig. 5A, AEDE-induced HO-1 expression was significantly inhibited by LY294005 and PD98059 but not by SP600125 and SB203580. Further, AEDE transiently increased the phosphorylation of Akt at early time (20 min), whereas ERK1/2 was activated by AEDE at later time (30-60 min) (Fig. 5B). These results suggest that PI3K/Akt and ERK-related signaling could constitute the major signal pathways in the regulation of HO-1 expression by AEDE.

Discussion

In this study, we demonstrate that AEDE inhibited iNOS expression via the induction of HO-1. Inhibition of iNOS activity in macrophages may thus represent an interesting target to treat various diseases including arthritis. In fact, administration of the selective inhibitors of iNOS has been reported to attenuate arthritis [7, 13]. Thus AEDE might reduce arthritis via inhibition of NO synthesis by affecting the iNOS expression level in macrophages.

Growing evidences have demonstrated that HO-1 exhibits anti-inflammatory activities by inhibiting production of pro-inflammatory mediators [31, 32], suggesting a potential therapeutic strategy for treating inflammatory diseases. Although the contribution of HO-1 products (ie, CO, biliverdin, and iron) has not been examined in this study, several studies point to HO-1-derived CO and biliverdin as the potential metabolite to combat inflammation [4, 8, 23, 26]. In particular, a recent report demonstrated that CO and biliverdin ameliorate murine collagen induced arthritis [4]. Moreover, HO-1 has been known not only to suppress inflammation but also to work as anti-oxidant and protect cells from oxidative stresses [5, 17, 34]. AEDE induced HO-1 and protected cells from LPS-derived cytotoxicity (Fig. 2 and 1D, respectively). Therefore, it is possible to suppose that AEDE protect cells from various oxidative stresses as well as exhibit anti-inflammatory effects.

In mammalian cells, PI3K/Akt and three MAPKs represented by ERK1/2, JNK, and p38 MAPK has been reported to be involved to some extent in HO-1 expression in response to diverse stimuli [15, 24]. Recently, Cheng and Lee described that MAPK and PI3K/Akt pathways are involved in the phosphorylation of Nrf2 to facilitate disassociation with Keap1 and nuclear translocation [6]. Thus AEDE-induced activities of PI3K/Akt and ERK might promote the disassociation from Keap1 and nuclear translocation of Nrf2,

although the effects of kinase inhibitors on Nrf2 were not examined in this study.

In conclusion, we demonstrated that AEDE inhibited NO release and iNOS expression in LPS-stimulated macrophages, and these effects are mediated by Nrf2-induced HO-1 expression. Our finding could help us to understand the active principle included in the roots of *A. japonica* and the molecular mechanisms underlying anti-inflammatory action of AEDE.

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초록 : LPS로 인한 RAW 264.7 세포의 염증반응에 미치는 achyranthoside E dimethyl ester의 효과

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Achyranthoside E dimethyl ester (AEDE)는 *Achyranthes japonica*에서 분리한 oleanolic acid glycoside이다. 본 연구에서는 대식세포에서 lipopolysaccharide (LPS)로 인한 nitric oxide (NO)의 생성에 미치는 AEDE의 효과를 관찰하고 그 작용 기전을 연구하였다. AEDE는 NO 생성과 inducible NO synthase (iNOS) 발현을 억제하였으며 세포에 독성을 유도하지 않았다. 또한 AEDE는 heme oxygenase-1 (HO-1)의 발현을 유도하였으며, HO-1 siRNA를 처리했을 때 AEDE가 iNOS의 발현을 억제하지 못하였다. AEDE는 HO-1의 발현에 관여하는 전사인자인 nuclear factor E2-related factor 2 (Nrf2)를 핵으로 이동시켰다. 한편 AEDE에 의한 HO-1의 발현은 phosphatidylinositol 3-kinase (PI-3K) 및 extracellular signal regulated kinase (ERK1/2) 억제제에 의해 감소되었으며, AEDE가 Akt와ERK1/2의 인산화를 유도하였다. 이상의 결과를 종합해보면, AEDE는 대식세포에서 PI-3K/Akt/ERK-Nrf2 신호전달과정을 통해 HO-1의 발현을 유도함으로써 NO와 같은 염증매개물질의 생성을 억제한다는 것을 알 수 있다. 이러한 연구결과는 AEDE가 항염증제로 사용될 수 있음을 시사한다.