Thesis for the Degree of Master of Science

αAL14, a novel peptide, has anti-inflammatory effects on LPS-stimulated RAW264.7 cells through inhibiting ERK/MAPK and NF-κB pathway.

by

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(신합성 펩타이드 αAL14의 ERK/MAPK와 NF-κB 신호전달 억제를 통한 LPS로 자극된 대식세포주 RAW264.7에서의 항염증 효과)

Advisor: Prof. Gun-Do Kim

by

Hyung-Wook Choi

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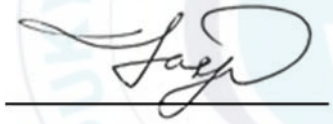

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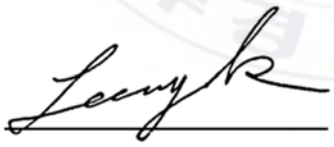

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Abstract

A novel model peptide, αAL14, has showed several bioactivities such as anti-angiogenic and anti-cancer activity in previous researches. In this study, we investigated anti-inflammatory properties of αAL14 in LPS-stimulated RAW264.7 macrophages. Although αAL14 had no significant effect on cell viability in RAW264.7 cells it decreased production of pro-inflammatory mediators, NO and PGE2 production in LPS-stimulated RAW264.7 cells. Moreover, αAL14 regulated expression of iNOS and COX-2 and decreased transcriptional level of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 compared to LPS only-treated cells. In MAPKs, only phosphorylation of ERK1/2 was suppressed in presence of αAL14, but not those of p38 and JNK/SAPK in LPS-stimulated RAW264.7 cells. Moreover, phosphorylation of NF-κB was inhibited and translocation of both p-ERK1/2 and NF-κB to nucleus was inhibited by αAL14 treatment. Phosphorylation and degradation of IκBα was suppressed in αAL14-treated RAW264.7 cells. Therefore, attenuated phosphorylation of ERK and NF-κB by αAL14 treatment can result in decreased expression of pro-inflammatory mediators such as NO, PGE2 and pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells. Consequently, we suggest that αAL14 possesses abilities to inhibit inflammatory responses in LPS-stimulated RAW264.7 macrophages.

1. Introduction

Inflammation, an innate immune system in hosts against antigens, involves removing antigens, wound healing and tissue repairing procedures [1, 2]. However, in chronic inflammation which is resulted from continuous exposure to antigens or self-antigens, inflammatory responses could lead to inflammatory diseases such as rheumatoid arthritis, dermatitis, type 2 diabetes, cancer and Alzheimer's disease [3]. Macrophages are a large proportion of inflammatory cells in chronic inflammation [4]. In inflammation, the macrophages are stimulated by antigens such as lipopolysaccharides (LPS) and they produce inflammatory mediators including nitric oxide (NO) and prostaglandin E2 (PGE_2), and pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 [5-7]. These pro-inflammatory mediators and cytokines contribute to recruiting and activating other immune cells during inflammatory process [8].

The mediators that can induce the inflammation are regulated by activation of mitogen-activated protein kinases (MAPKs) and transcription factors, nuclear factor-kappa B (NF-κB) [9]. MAPKs include extracellular signal-regulated kinases (ERK), p38, and c-jun N-terminal kinase (JNK). Although each sort of MAPKs has distinct roles in different signaling pathways activated by stimuli, they can control expression of main inflammation-related genes by phosphorylating their substrates in immune cells [10]. NF-κB is the most canonical transcription factor stimulated by pathogen associated molecular patterns (PAMPs) such as LPS [11]. In NF-κB

signaling pathway, NF-κB is activated by LPS through phosphorylation and subsequently translocated into nucleus and promotes transcription of pro-inflammatory cytokines and mediators [8, 12].

Peptides that consist of up to 50 amino acids without tertiary structure function as hormones, neurotransmitters, growth factors and antibiotics [13]. Based on their diverse functions, several peptides have been used with or without modification in their sequence to treat diseases such as cancer, obesity and inflammation [14, 15]. Many researches proved that peptides play an important role in regulating immune response with specific targets involved in immune cell activation [16]. Newly synthesized peptide, αAL14, is composed of 14 amino acids including rich Lysine residues, which present α-helical structure. A mother sequence of αAL14 was derived from abalone and it has been modified in its length and several resides. Previous study, it revealed that αAL14 can inhibit angiogenesis and gastric cancer cell growth (not published data). And it is well known that MAPKs and NF-κB, which are crucial factors in inflammation, play key roles in angiogenesis and cancer progression [17, 18]

Therefore, in this study, we hypothesized that αAL14, a new peptide, could inhibit MAPKs and NF-κB pathway in RAW264.7 inflammatory model, so it was investigated whether new peptide model αAL14 can regulate inflammatory response in Raw264.7 murine macrophages. In addition, molecular targets of αAL14 were identified regarding immune cell activation by LPS stimulation.

2. Materials and methods

2.1 Peptide synthesis

αAL14 was synthesized and purified as described previously [19]. Briefly, αAL14 was synthesized by Peptron Inc. (Daejeon, Korea) with a purity grade of > 95 % using N-(9-Fluorenylmethoxycarbonyl) (Fmoc) solid phase with ASP48S, and purified by reverse phase high performance liquid chromatography (HPLC). Prediction of peptide structure was performed as described [5]. A theoretical isoelectric point (pI) and helical wheel diagram were developed using ExPASy's ProtParam server (http://www.expasy.org) and EMBOSS pepwheel sequence analysis program (European Bioinformatics Institute, Cambridge, UK), respectively [20].

αAL14 consists of 14 amino acids having a sequence AAWKLLKALAKAAL. The schematic structure of this novel peptide is described in Fig 1.

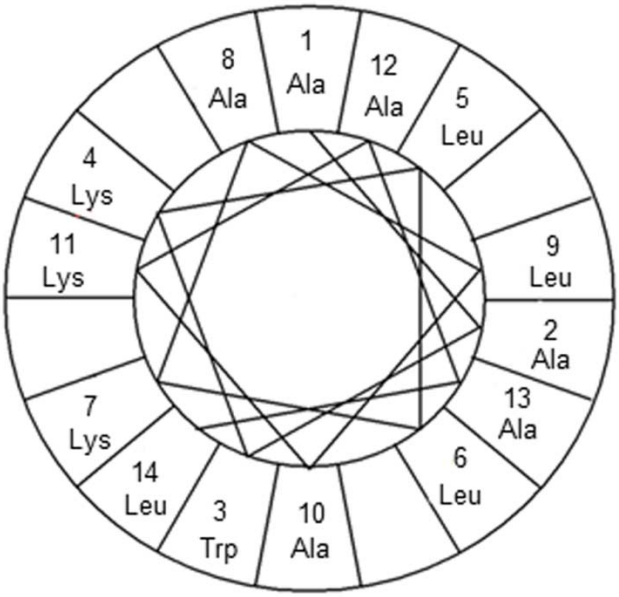

Figure 1. Schematic structure of αAL14. αAL14 has a amphiphilic α-helical formation composed of hydrophilic residues (K4, K7 and K11) on one side and hydrophobic residues (A1, A2, L5, L6, A8, A9, A10, A12, A13 and L14) on the other side.

2.2 Reagents

The Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Mediatech Corning (Manassas, VA, USA). Lipopolysaccarides (LPS) from Escherichia coli O111:B4, Griess reagent, Triton X-100, and 2-mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit primary antibodies (iNOS, COX2, p-ERK1/2 (Thr202/Tyr204), p38, p-p38 (Thr180/Tyr182), JNK/SAPK, p-JNK/SAPK (Thr183/Tyr185), NF-κB p65, p-NF-κB p65 (Ser536), p-IκBα (Ser32), GAPDH and -Histone H3), anti-IκBα mouse antibody, HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies, and anti-rabbit IgG (H+L), F(ab')2 fragment (Alexa Fluor®488 conjugate) were purchased from Cell Signaling Technology Inc. (Denver, MA, USA). Anti-ERK1/2 rabbit antibodies and rabbit normal serum were obtained from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). DAPI (4', 6-diamidino-2-phenylindole) was purchased from Roche Diagnostics (GmBH, Mannheim, Germany) and formaldehyde was bought from JUNSEI Chemical Co., (Tokyo, Japan). ProLong® Gold Anti-fade Reagent (Invitrogen, Eugene, OR, USA) was used.

2.3 Cell culture

Murine macrophage RAW264.7 cells (American Tissue Culture Collection, Manassas, VA, USA) were incubated with DMEM containing 10% FBS and 1%

penicillin (100 U/ml)/streptomycin (100 μg/ml). Cells were maintained at 37℃ with a humidified condition of 5% CO_2.

2.4 Cell viability assay

To determine the effect of αAL14 on the cell viability of RAW264.7 cells, 1×10^4 of cells were seeded in each well of 96-well cell culture plates. The cells were treated with several concentrations (1, 5, 10 and 20 μM) of αAL14 for 24 h. After incubation, medium in wells was changed to fresh medium. 10 μl of EZ-cytox Cell Viability Assay Solution WST-1® (Daeil Lab Service, Gyeonggi, Korea) was added to each well and the cells were incubated for 3 h. Then, the optical density (OD) at 460 nm was measured by a microplate reader (Molecular Devices, Sunnyvale, CA, US).

2.5 Nitric oxide detection assay

Cells were seeded in 24-well cell culture plates (5×10^4 cells/well) and pre-treated with 1, 5, and 10 μM of αAL14 for 2 h. 1 μg/ml LPS was added for stimulation and cells were incubated for 24 h. To determine NO production, 100 μl of the culture supernatant were transferred to 96-well plates. The same volume of Griess reagent was added to the supernatant and incubated for 10 min at room temperature in dark condition. Absorbance was measured at 540 nm using the microplate reader.

2.6 Enzyme-linked immunosorbent assay (ELISA) of PGE_2

An ELISA kit for detecting PGE_2 was purchased from R&D system (Minneapolis, MN, USA). Cells (5×10^4 cells/well) were plated on 24-well cell culture plates and pre-treated with αAL14 for 2 h and LPS was added. After further incubation for 24 h, media were collected and the assays were performed according to the manufacturer's protocols. Absorbance was measured by the microplate reader.

2.7 Western blot analysis

RAW264.7 cells were pre-treated with αAL14 for 2 h before stimulated with or without LPS (1 μg/ml) for 3 h or 24 h to perform Western blot analysis. For whole cell lysis, cells were extracted by cell lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1 % Triton X-100, 1 % Deoxycholate, 0.1 % SDS) (Intron Biotechnology, Gyenggi, Korea). For preparation of nuclear extracts, cultured cells were harvested and lysed using NE-PER® nuclear and cytoplasmic extraction reagents (Life technology, Carlsbad, CA, USA) with manufacturer's protocol. The protein concentration in the cell lysates was determined by Bradford reagent (Biosesang, Seongnam, Korea). Equal volume of prepared proteins were resolved by 12% SDS-PAGE and then transferred to a nitrocellulose membrane (Pall Life Sciences, Ann Harbor, MI, USA). The membrane was blocked with 1× PBST buffer containing 5 % skim milk for 1 h and then incubated with primary

antibodies overnight at 4℃. The membrane was incubated with anti-rabbit or anti-mouse IgG second antibodies conjugated with HRP for 1 h at room temperature. The membrane was developed by an enhanced chemiluminescent (ECL®) detection solution (Pierce, Rockford, IL, USA). Band intensities were quantified using Image J.

2.8 Immunofluorescence staining

Cells were seeded on cover-glass bottom dishes (SPL Lifesciences, Pocheon, Korea) and pre-treated with 5 μM of αAL14 for 2 h. Then the cells were stimulated by LPS for 3 h or 24 h to induce the expression of p-ERK1/2 or NF-κB, respectively. After incubation, the cells were stained by using 1 μg/ml DAPI and incubated for 15 min at 37℃, washed with PBS buffer, and fixed with 4 % formaldehyde for 15 min at room temperature. The cells were blocked with blocking solution (5 % rabbit normal serum containing 0.3 % Triton X-100 in 1× PBS) for 1 h in dark condition. The cells were incubated with the anti-ERK1/2 (Thr202/Tyr204) or -NF-κB p65 primary antibody at 4℃ for overnight. After the reaction, the cells were washed with 1× PBS and then reacted with anti-rabbit IgG (H+L), F(ab')2 fragment (Alexa Fluor®488 conjugate) for 1 h at room temperature in dark. The cells were washed with 1× PBS and mounted using ProLong® Gold Anti-fade Reagent. The cells were observed by using Carl Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

2.9 Reverse transcription (RT)-PCR of cytokine transcripts

Total RNA was extracted by 2-mercaptoethanol and isolated using RNeasy plus mini kit (Qiagen, Venlo, KJ, Netherlands). The concentration of total RNA was determined by nanodrop (MECASYS, Daejeon, Korea) and 2 μg of RNA was synthesized to cDNA using cDNA kit (Genetbio, Daejeon, Korea). Same volume of cDNA was amplified by PCR using specific primers shown in Table 1. The amplified PCR products were observed on 2 % agarose gel. Band intensities were measured using Image J.

Table 1. The primer sequences for RT-PCR

Genes	5′-Forward primer-3′	5′-Reverse primer-3′
TNF-α	ACGGCATGGATCTCAAAGAC	CGGACTCCGCAAAGTCTAAG
IL-1β	CAGGCAGGCAGTATCACTCA	AGGCCACAGGTATTTTGTCG
IL-6	AACGATGATGCACTTGCAGA	CTCTGAAGGACTCTGGCTTTG
GAPDH	AACTTTGGCATTGTGGAAGG	CACATTGGGGGTAGGAACAC

2.10 Statistical analysis

GraphPad Prism 6.0 was used to determine the statistical significance of data. Results were expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with post-hoc tests and Dunnett's multiple comparison tests

was used to assess differences between non-treated or LPS only-treated group and αAL14-treated groups. $p < 0.05$ was considered as statistical significance. All experiments were performed in triplicates independently.

3. Results

3.1 Effects of αAL14 on cell viability of RAW264.7 cells

To determine cytotoxic effect of αAL14 on RAW264.7 cells, cell viability was measured by WST-1 assay. The cells were treated various concentrations of αAL14 for 24 h. As shown in Fig. 2, αAL14 did not affect the cell viability at concentrations less than 10 μM. Therefore, based on this result, the concentrations of αAL14 less than 10 μM were used to identify its anti-inflammatory effects to exclude the cytotoxic effect of αAL14 in RAW264.7 cells.

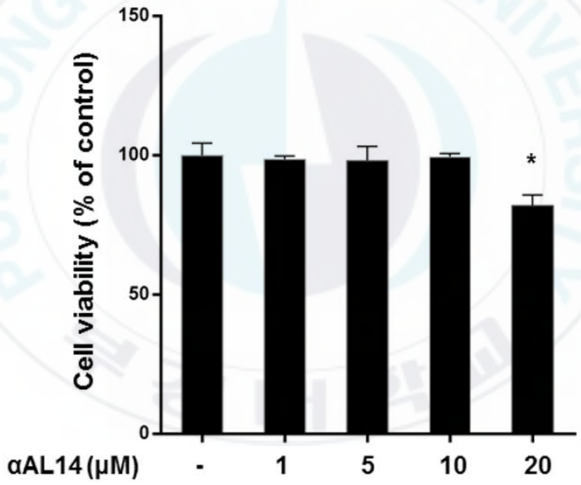

Figure 2. Effects of αAL14 on cell viability of RAW264.7 cells. Cells were treated with 1, 5, 10, and 20 μM of αAL14 for 24 h. Cell viability was determined by WST-1. Values in the graph indicate mean ± SD from three independent experiments. (*p<0.01 vs. non-treated group)

3.2 Effects of αAL14 on NO production and PGE_2 secretion in RAW264.7 cells

RAW264.7 cells were pre-treated with or without αAL14 for 2 h and stimulated by LPS for 24 h. While NO production was increased in LPS-stimulated cells compared to non-treated cells, in αAL14-treated cells which were stimulated with LPS, NO production was significantly reduced in a dose-dependent manner (Fig. 3A). In addition, expression of iNOS was down-regulated in both αAL14 and LPS-treated cells compared to LPS only-treated cells (Fig. 3B). As continuous PGE_2 secretion from immune cells causes chronic inflammation, effects of αAL14 on PGE_2 secretion was investigated. PGE_2 secretion in LPS-stimulated macrophages was decreased when the cells were treated with αAL14 in a dose-dependent manner (Fig. 3C). Likewise, the expression of COX-2 was down-regulated by the same concentrations of αAL14 compared to LPS only-treated cells (Fig. 3D). Therefore, these results suggest that αAL14 can inhibit production of NO and PGE_2 through suppressing iNOS and COX-2 expression in LPS-stimulated RAW264.7 cells.

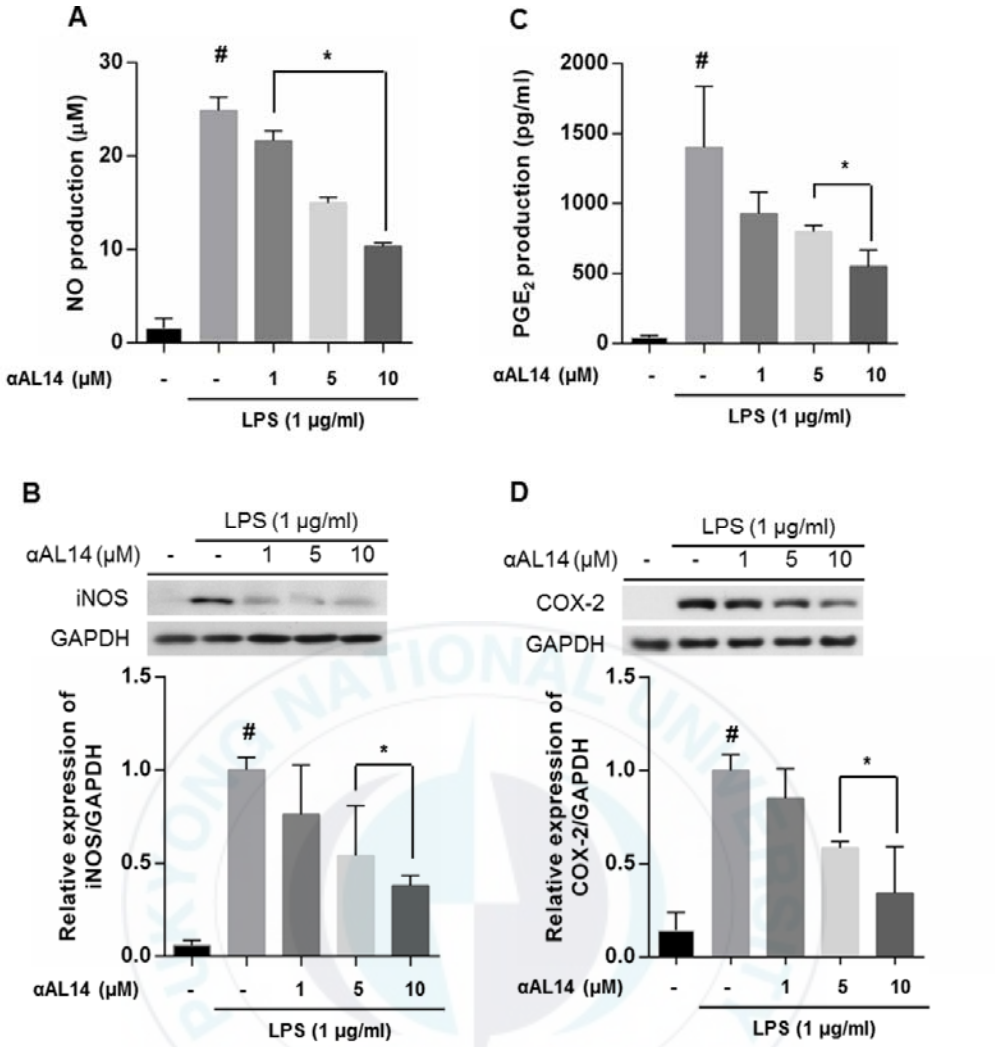

Figure 3. Effects of αAL14 on NO and PGE2 secretion in LPS-stimulated RAW264.7 cells. Cells were pre-treated with indicated concentrations of αAL14 and stimulated by LPS for 24 h. Production of NO was determined by Griess reagent with cell supernatant (A) and expression of iNOS was determined by western blot in cell whole lysates (B). Values indicates mean ± SD (n=3; #p<0.01 vs. non-treated group and *p<0.01 vs. LPS only-treated group). (C) The secretion level of PGE2 was measured by ELISA kit. Values in the graphs is mean ± SD (n=3; #p<0.01 vs. non-treated group and *p<0.01 vs. LPS only-treated group). (D) COX-2 expression in whole lysate was determined by western blot. Each value in the graphs is mean ± SD (n=3; #p<0.01 vs. non-treated group and *p<0.01 vs. LPS only-treated group).

3.3 Effects of αAL14 on mRNA level of pro-inflammatory cytokines

To investigate effects of αAL14 on transcriptional level of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, RT-PCR were performed. In LPS-stimulated RAW264.7 cells, mRNA expression of all three pro-inflammatory cytokines was significantly increased. However, when LPS-activated cells were treated with αAL14, the transcripts of these cytokines were decreased in a dose-dependent manner (Fig. 4).

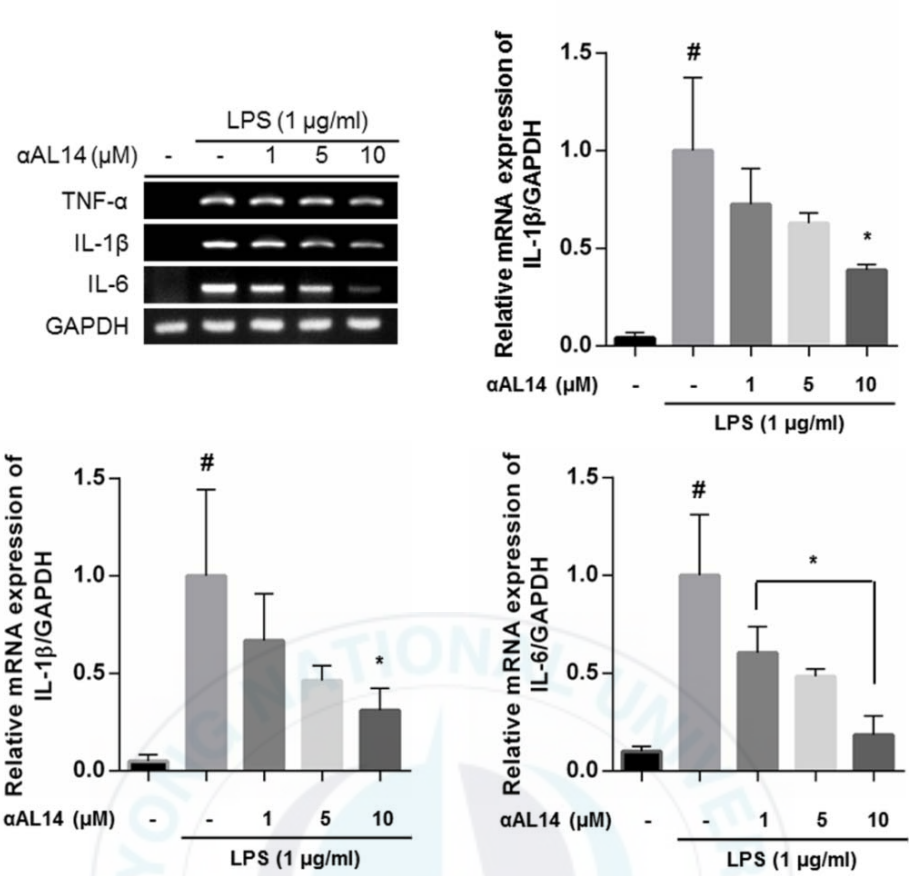

Figure 4. Effects of αAL14 on mRNA expression of pro-inflammatory cytokines. Cells were pre-treated with 1, 5, and 10 μM of αAL14 for 2 h before activation with LPS for 6 h. cDNA was synthesized from total RNA and specific primers were used to detect each gene. GAPDH was used as a loading control. The intensity of each PCR products was analyzed by Image J software. The data in the graphs are mean ± SD (n=3) (#p<0.01, vs. non-treated group and *p<0.01, vs. LPS-stimulated cells)

3.4 Effects of αAL14 on LPS-induced phosphorylation of MAPKs

To elucidate whether αAL14 can affect activation of MAPK family which play important roles in inflammatory responses, phosphorylated ERK, p38 and JNK were investigated. Phosphorylation of these three proteins was increased in LPS only-treated RAW264.7 cells. When αAL14 treated RAW264.1 cells, the phosphorylation of ERK1/2 was significantly decreased but those of p38 and JNK/SAPK was not affected (Fig. 5). Moreover, distribution of phosphorylated ERK1/2 away from nucleus was observed in both LPS- and αAL14-treated cells compared to LPS only-treated cells (Fig. 6).

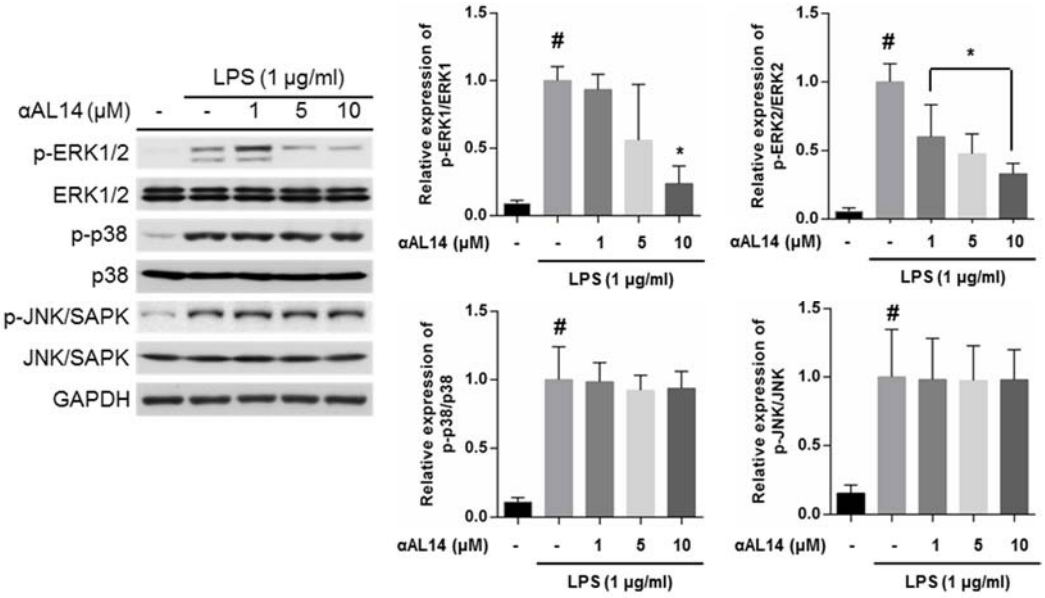

Figure 5. Effects of αAL14 on phosphorylation of MAPKs in LPS-activated RAW264.7 macrophages. Cells were pre-treated with 1, 5, and 10 μM of αAL14 for 2 h before activation with 1μg/ml of LPS for 6 h. Total proteins and phosphorylated proteins were detected using western blot in cell whole lysates. GAPDH was used as a loading control. The intensity of each protein was analyzed by Image J software. The data in the graphs are mean ± SD (n=3). #p<0.01, *vs*. non-treated group and *p<0.01, *vs*. LPS-stimulated cells.

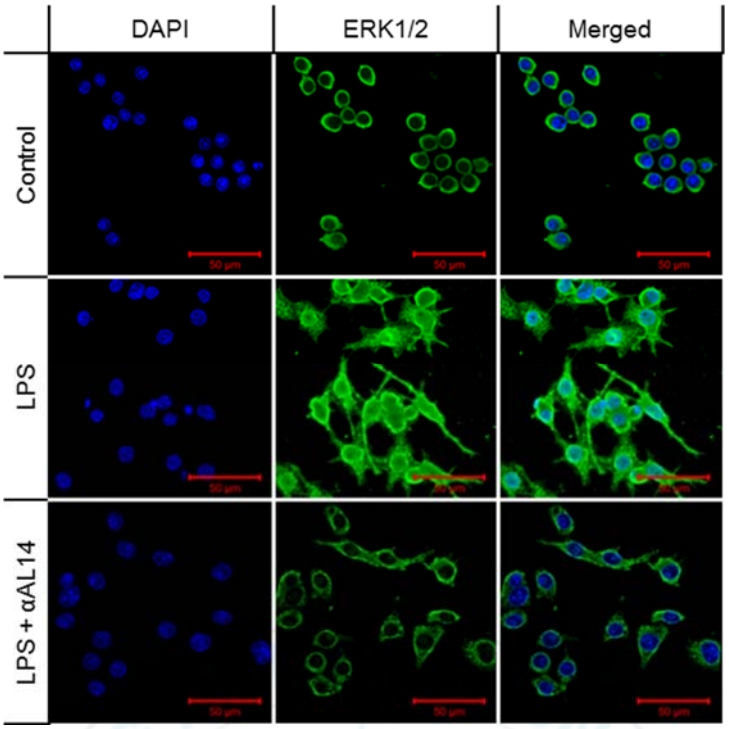

Figure 6. Effect of αAL14 on nuclear translocation of ERK1/2. Cells were pre-treated with 10 μM of αAL14 for 2 h before activation with 1μg/ml of LPS for 3 h. It was detected by immunofluorescence staining and observed by confocal microscopy with 10 μM of αAL14 (blue: DAPI and green: ERK1/2). Scale bars = 50 μm.

3.5 Effects of αAL14 on LPS-activated NF-κB signal transduction

Because NF-κB signal transduction is important for inflammation, effects of αAL14 on phosphorylation of NF-κB were investigated. When LPS-stimulated RAW264.7 macrophages were treated with αAL14, phosphorylation of NF-κB was decreased compared to LPS only-treated cells in a dose-dependent manner (Fig. 6A). Expression and phosphorylation level of NF-κB regulator, IκBα, were determined by western blot analysis with whole lysate. As a result, phosphorylated IκBα was increased and its total form was decreased in LPS only-stimulated cells. However, in contrast to LPS-stimulated cells, phosphorylated IκBα was decreased and total form of IκBα was increased in αAL14-treated cells (Fig. 6B). Moreover, expression level of NF-κB in nucleus was decreased by αAL14 treatment in LPS-stimulated cells (Fig 6C) and it was confirmed by immunofluorescence staining of NF-κB. NF-κB was dominantly distributed in cytosol in αAL14-treated cells, whereas it distributed throughout both nucleus and cytosol in LPS only-treated cells (Fig 6D). Therefore, these data suggest that αAL14 treatment reduces activation and translocation of NF-κB into nucleus by enhancing expression of IκBα in LPS-stimulated RAW264.7 cells.

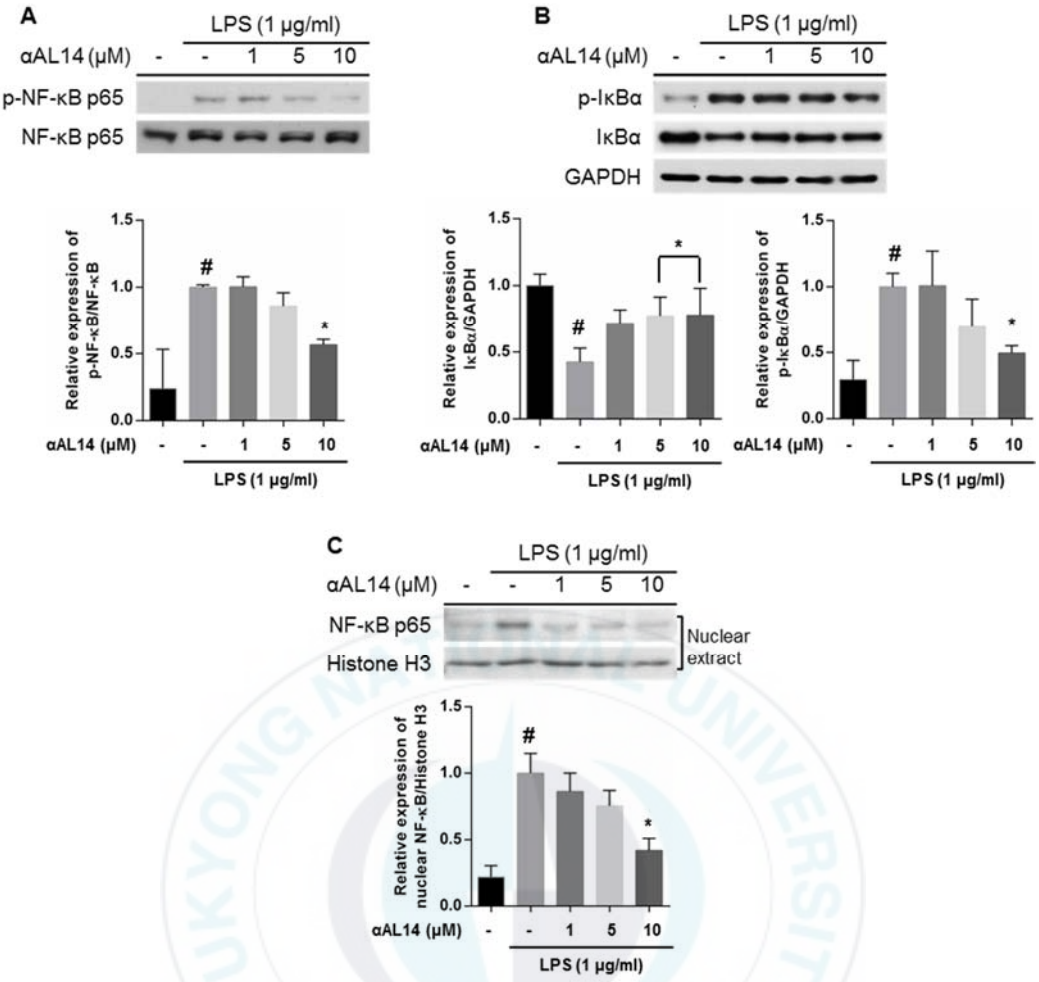

Figure 7. Effects of αAL14 on NF-κB pathway in LPS-stimulated RAW264.7 cells. Cells were pre-treated with αAL14 for 2 h before activation with LPS for 24 h. (A and B) Both total form and phosphorylation form of NF-κB or IκBα were investigated using cell whole lysates. The data in the graphs are mean ± SD (n=3; #p<0.01, vs. non-treated group and *p<0.01, vs. LPS-stimulated cells). (C) To evaluate nuclear translocation of NF-κB, nuclear NF-κB was examined. GAPDH and histone H3 were shown as loading control. The data in the graphs are mean ± SD (n=3; #p<0.01, vs. non-treated group and *p<0.01, vs. LPS-stimulated cells.

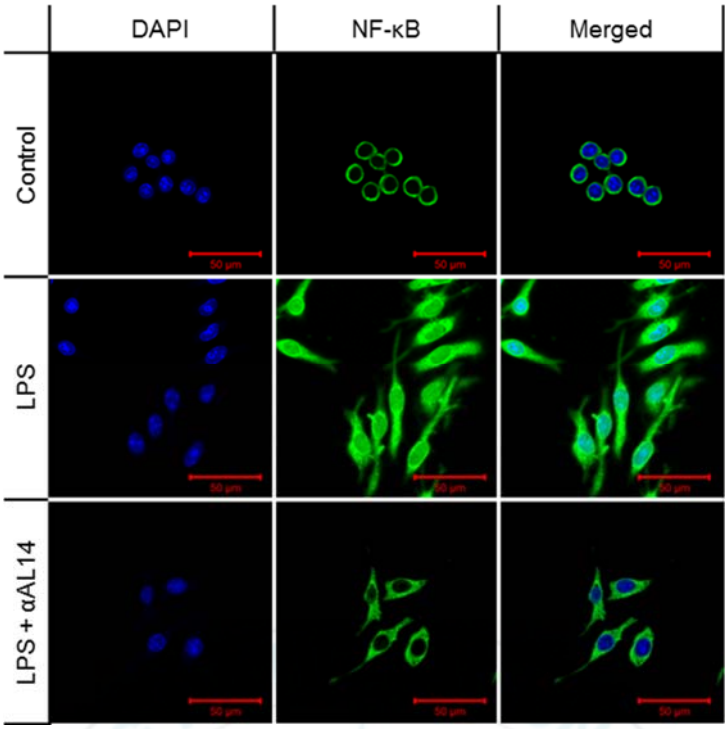

Figure 8. Effects of αAL14 on nuclear translocation of NF-κB in LPS-stimulated macrophages. Cells were pre-treated with 10 μM of αAL14 for 2h, and then activated with LPS for 24h. The nuclear translocation was detected by immunofluorescence staining with 10 μM of αAL14 (blue: DAPI and green: NF-κB). Scale bars = 50 μm.

4. Discussion

It has been revealed that αAL14, a novel model peptide, has anti-cancer and anti-angiogenesis effects in our previous studies (not published). In this study, we focused on identifying anti-inflammatory properties of αAL14. It is firstly evaluated whether αAL14 can affect production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in LPS-stimulated RAW264.7 murine macrophages because they are important pro-inflammatory mediators during inflammatory response [21, 22]. Compared to LPS only-treated cells, the production of NO and PGE_2 was reduced by αAL14 without any cytotoxic effects on RAW264.7 cells. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are involved in synthesis of NO and PGE_2 [5, 23]. It has been revealed that iNOS and COX-2 which play critical roles in inflammatory response are upregulated in LPS-stimulated RAW264.7 murine macrophages [24-26]. Therefore, our results suggest that αAL14 has an ability to attenuate production of NO and PGE_2 through down-regulation of expression of iNOS and COX-2 because expression level of these two proteins were significantly suppressed in response to αAL14 treatment in LPS-stimulated RAW264.7 cells. Moreover, LPS-stimulated macrophages produce pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 as well, which activate various signaling pathways involved in inflammation [7]. However, in LPS-stimulated RAW264.7 cells, mRNA level of TNF-α, IL-1β and IL-6 was decreased by αAL14 treatment, our

data suggest that αAL14 is capable of attenuating inflammatory response through regulating production of various pro-inflammatory factors including NO, PGE_2, and pro-inflammatory cytokines. Based on these results, it was hypothesized that αAL14 can regulate several cellular signaling involved in production of pro-inflammatory cytokines in macrophages. Mitogen-activated protein kinases (MAPKs) consist of ERK1/2, p38, and JNK, which have various functions in innate immunity such as activating co-activators and transcription factors, and stabilizing pro-inflammatory cytokines [27, 28]. MAPKs are activated by several extracellular signals such LPS and cytokines via each combined or separated pathways [29]. Guha and Mackman demonstrated that LPS stimulation induces activation of ERK cascades through sequential phosphorylation of kinases involved in, which finally leads to phosphorylation of ERK1/2 in macrophages [30]. Activated ERK1/2 promotes phosphorylation of downstream proteins that contribute to control inflammatory responses or acts as a transcription factor in nucleus to induce expression of inflammation relating genes [31]. According to our results, αAL14 only affected phosphorylation level of ERK1/2 though LPS induced phosphorylation of three different sorts of MAPKs, ERK1/2, p38, and JNK. It suggests that αAL14 is more effective to inhibit activation of ERK1/2 but neither p38 nor JNK. Moreover, it was observed that phosphorylated ERK 1/2 was localized away from nucleus by αAL14 treatment in LPS-stimulated RAW264.7 cells, which confirms that αAL14 has inhibitory effects on ERK activities in terms

of regulation of immune response. Therefore, these date suggest that αAL14 can regulate immune response stimulated by LPS by inhibiting ERK activities.

NF-kB signal transduction is a canonical to control expression of pro-inflammatory mediators such as iNOS and COX-2, and pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β [32]. In basal state, NF-κB exists in cytosol as an inactivated form and masked by inhibitor of NF-κB (IκB) α whereas, in LPS-stimulated cells, IκBα is phosphorylated by IκB kinase β (IKKβ) and degraded by proteasome [33]. Thus it is suggested that αAL14 is capable of protecting degradation of IκBα from proteasome by inhibiting its phosphorylation because phosphorylation level of IκBα was decreased by αAL14 treatment in in LPS-stimulated RAW264.7 cells. In addition, it has been known that departed NF-κB from IκBα can translocate to nucleus and translocated NF-κB continuously binds to κB-site in DNA and recruits transcription machinery [32, 34, 35]. When αAL14 treated LPS-stimulated macrophages, nuclear NF-κB is decreased, thus it is suggested that αAL14 can regulate LPS-induced production of major inflammatory cytokines, TNF-α, IL-6, and IL-1β by inhibiting NF-κB activation and translocation. In light of unbalanced production of pro-inflammatory cytokines is implicated in many diseases [36], our results show that αAL14 has potential for being developed as an effective immunoregulator.

In conclusion, as the first research on anti-inflammatory properties of αAL14 in RAW264.7 cells, our results suggest that αAL14 has ability to decrease pro-

inflammatory mediators including NO, PGE2, pro-inflammatory cytokines via inhibition of ERK and NF-κB pathway. Therefore, our study suggests that this novel peptide, αAL14 potentiates to be a novel anti-inflammatory application.

5. Acknowledgement

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6. 국문초록

이전 연구 결과들에서 신합성된 펩타이드인 αAL14 의 신생혈관생성 억제 및 항암 효과와 같은 생리활성들이 연구된 바 있으며, 본 연구에서는 LPS 로 유도된 RAW264.7 대식세포에서의 염증 억제 효과를 확인하였다. αAL14 는 RAW264.7 세포주에서 세포독성을 거의 보이지 않았으며, NO 와 PGE_2 와 같은 염증성 인자들의 생성을 크게 억제하였다. 또한 iNOS 와 COX-2 의 발현이 αAL14 에 의해 감소하였고, TNFα, IL-1β, IL-6 와 같은 염증성 사이토카인의 mRNA 발현 또한 αAL14 에 의해 조절됨을 확인하였다. MAPK 의 경우, p38 과 JNK/SAPK 의 인산화는 변화가 없었지만 ERK1/2 의 인산화가 αAL14 에 의해 유의적으로 감소함을 확인하였다. 또한 인산화된 ERK1/2 의 핵으로의 translocation 이 억제되었으며, NF-κB 의 경우 그 인산화가 감소하였고, translocation 또한 억제됨을 확인하였다. 그리고 IκBα 의 인산화 및 분해가 억제되는 것을 확인하였다. 이를 통해, αAL14 에 의해 저해된 ERK 와 NF-κB 의 인산화가 NO, PGE_2 와 같은 염증성 인자 및 염증성 사이토카인의 발현을 감소시킨다는 것을 확인하였다. 결과적으로 αAL14 가 LPS 로 유도된 RAW264.7 대식세포에서 염증 반응을 억제시키는 활성을 가짐을 확인하였다.

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