2017 2(1) 17-23 Submission: 2017/04/30, Peer review: 2017/05/10, Accepted: 2017/06/25, Published: 2017/06/30

International journal of sport

Publication state: Japan
ISSN: 2423-8287

Publisher: J-INSTITUTE
Website: http://www.j-institute.jp

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Peer reviewer
E-mail: editor@j-institute.jp

http://dx.doi.org/10.22471/sport.2017.2.1.17

Anti-Inflammatory Effect of Cynanchone a in Lipopolysaccharide-Induced RAW 264.7 Cells

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Abstract

It has been reported that Cynanchum wilfordii exhibits anti-oxidant, and anti-cancer activities. However, little is known about biological activity of cynanchone A, an acetophenone isolated from C. wilfordii. In the present study, we investigated the anti-inflammatory effect and the underlying molecular mechanisms of cynanchone A in lipopolysaccharide (LPS)-induced RAW 264.7 cells. During the LPS-induced inflammatory process in RAW 264.7 macrophages, nitric oxide (NO) and prostaglandin E2 (PGE2) productions increased. Cynanchone A significantly decreased the protein levels of NO and PGE2 in LPS-treated RAW 264.7 macrophages. Moreover, cynanchone A significantly reduced the elevated the expression levels of inducible nitric oxide (iNOS), cyclooxygenase-2 (COX-2) and tumor necrosis factor-a(TNF-a) in LPS-stimulated RAW264.7 cells. Consequently, cynanchone A exerted an anti-inflammatory action through inhibiting nuclear factor-kappa B (NF-κB) activation. Taken together, our results show that the anti-inflammatory properties of cyanchone A are involved in the down-regulating pro-inflammatory cytokines and regulating NF-kB signal pathway in LPS-stimulated RAW264.7 cells.

[Keywords] ***Cynanchum Wilfordii, RAW264.7, Anti-Inflammatory, Anti-Oxidant, Cyanchone A***

1. Introduction

Inflammation is a process that involves multiple factors that act in concert. The ingress of leukocytes into sites of inflammation is an important aspect of the pathogenesis of inflammatory conditions[1]. For example, macrophages are recruited to inflammatory sites, and are activated by various signals that stimulate many intracellular cascades of cytokines and chemokines[2]. In macrophages, lipopolysaccharide (LPS), a well-known endotoxin, increases the productions of inflammatory cytokines, such as, tumor necrosis factor-α (TNF-α) and interleukin-6, and inflammatory mediators, such as, nitric oxide (NO) and prostaglandin E2 (PGE_2), which are synthesized by inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively[3][4].

NF-κB is a transcriptional factor that plays a pivotal role in immune and inflammatory responses via the regulation of genes that encode pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes like COX-2 and iNOS[5][6]. Under normal conditions, NF-κB is sequestered in the cytoplasm as an inactive complex, due to binding with IκB proteins [7]. In the classic pathway, activation of NF-κB, especially the most abundant form, p50/p65 heterodimer, depends on the phosphorylation of its endogenous inhibitor IκB, mainly by IκB kinases (IKKs)[8][9]. This leads to ubiquitination and proteasomal degradation of IκB. The liberated NF-κB dimer then translocates to the nucleus, where it activates specific target genes[10][11]. Several studies have reported that the activation of NF-κB is triggered by mitogen-activated protein kinases (MAPKs), including extracellular signal-

regulated protein kinases 1/2, p38 MAPK kinase, and JNK[12]. Other reports have shown that the negative regulation between NF-κB and MAPK[13]. The relationship between NF-κB and MAPKs are complex and depends on the cell type and stimulus.

The roots of *Cynanchum wilfordii* have traditionally been used to help liver and kidney, to strengthen the bone and muscle, and to invigorate in Korea. In previous studies have shown that the extract and fractions of this herb have various pharmacological activities, including the effect of scavenging against free radicals, enhancing immunity, reducing high serum cholesterol, and having anti-tumor activity[14]. Cynanchone A is acetophenone isolated from roots of *C. wilfordii*. Since the first discovery of Cyanachone A in 1999, these biological effects were still poorly understood. Therefore, as a part of our ongoing screening program to evaluate the anti-inflammatory potentials of natural compounds, we selected Cynanchone A, and investigated its anti-inflammatory effects and the mechanism involved in RAW264.7 macrophage cells, which can be stimulated by LPS to mimic conditions of infection and inflammation.

2. Materials and Methods

2.1. Cell culture and sample treatment

The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37°C in DMEM medium supplemented with 10 % FBS, penicillin (100 units/ml), and streptomycin sulfate (100 µg/ml) in a humidified atmosphere of 5% CO_2. Cells were incubated with Cynanchone A at concentrations of 25, 50, and 100 µM, and then stimulated with LPS (1 µg/ml) for the indicated time.

2.2. Measurement of cell viability by MTT assay

RAW 264.7 ($1x10^4$/well) cells viability studies were performed in 96-well plates. Cynanchone A was dissolved in DMSO, and the DMSO was added to all plates to compensate the same volume of DMSO. After overnight incubation, the test material was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 µl of FBS-free medium containing 5 mg/ml of MTT. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue that formed in the cells was dissolved in DMSO 100 µl. The optical density was measured at 540 nm.

2.3. Measurement of nitrite in culture media

RAW 264.7 cells were plated at 2.5×10^5 cells/ml in 24 well-plates and then incubated with or without LPS (1 µg/ml) in the absence or presence of various concentrations (25, 50, and 100 µM) of Cynanchone A for 24 h. The nitrite accumulated in culture medium was measured as an indicated of NO production based on the Griess reaction. Briefly, 100 µl. of cell culture medium was mixed with 100 µl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl], incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured in a microplate reader (Perkin Elmer Cetus, Foster City, CA, USA). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was measured with the serial dilution standard curve of sodium nitrite.

2.4. Determination of PGE2, TNF-α, IL-6, and IL-1 productions

RAW 264.7 cells were pretreated with Cynanchone A (25, 50, and 100 µM) for 1 h and then stimulated with LPS (1 µg/ml) for 24 h. Levels of PGE2, TNF-α, interleukin (IL)-6, and interleukin (IL)-1 in the culture media were quantified using ELISA kits (R&D Systems, Minneapolis, MN. USA).

2.5. Protein extraction and western blot analysis

RAW 264.7 cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phe-

nylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride, and 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated with 20 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacture's instruction. Forty micrograms of cellular protein was electroblotted onto a polyvinylidene fluoride (PVDF) membrane following separation on a 10% SDS-polyacrylamide gel electrophoresis. The membrane was incubated overnight with blocking solution (5% skim milk) at 4 ℃, Followed by incubation for 4 h with a primary antibody. The membrane was washed four times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1:1000 dilution secondary antibody for 1 h at room temperature. The membrane was were again washed three times with TTBS, and then developed by enhanced chemiluminescence (Amersham Life Science.)

2.6. Statistical analysis

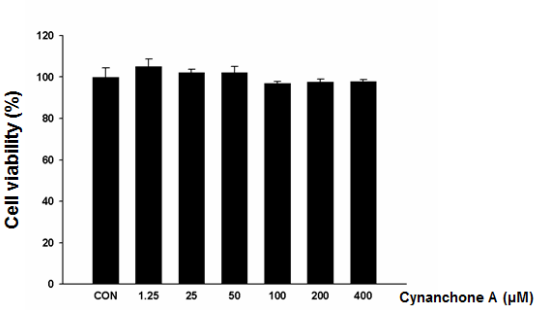
Results are expressed as the mean ± S.D. of triplicate experiments. Statistical significant values were compared using ANOVA and Dunnett's post-hoc test, and *p*-values of less than 0.05 were considered as significant.

3. Results

3.1. Effects of cynanchone A on on LPS-induced NO and PGE2 production in RAW 264.7 macrophages

The cytotoxic effects of cynanchone A were also evaluated in the presence of LPS using MTT assays, and this compound had cytotoxicity at the concentrations of 100 μM <Figure 1>. We investigated the inhibitory effects of cynanchone A on the LPS-induced productions of the inflammatory mediators NO and PGE_2 in RAW 264.7 cells. Neither LPS nor samples were added to the control (CON) group. As shown in <Figure 2>, LPS (1 μg/ml) increased NO production by approximately 12-fold, whereas pretreatment with cynanchone A (25, 50, or 100 μM) markedly reduced LPS-induced NO production in a dose-dependent manner. As shown in <Figure 2>, cynanchone A also dose-dependently inhibitedPGE_2 production by LPS.

Figure 1. Dose responses effects of cyanchone A on the viability of RAW 264.7 cells.



3.2. Inhibitory effects of cynanchone A on LPS-induced proteins and on the mRNA expressions of iNOS and COX-2

In present study, cynanchone A whether can reduce the LPS-induced NO and PGE_2, we examined their expression levels by Western blotting and RT-PCR. In unstimulated RAW 264.7 cells, iNOS and COX-2 protein levels were undetectable. However, in response to LPS, their expressions were markedly upregulated. Furthermore, cynanchone A significantly inhibited LPS-induced iNOS and COX-2 in a dose-dependent manner <Figure 3>.

Figure 2. Inhibitory effects of cynanchone A on LPS-induced NO and PGE2 production in RAW 264.7 macrophages. Each value indicates the mean ± SD and is representative of the results obtained from three independent experiments (#p<0.05 compared with the control; *p<0.05 and **p<0.01 compared with cells cultured with 1 μg/ml LPS).

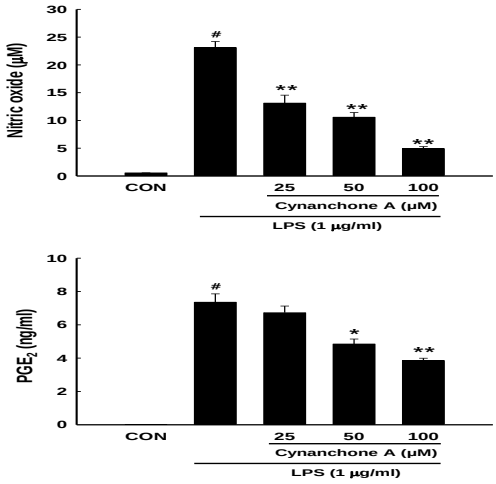
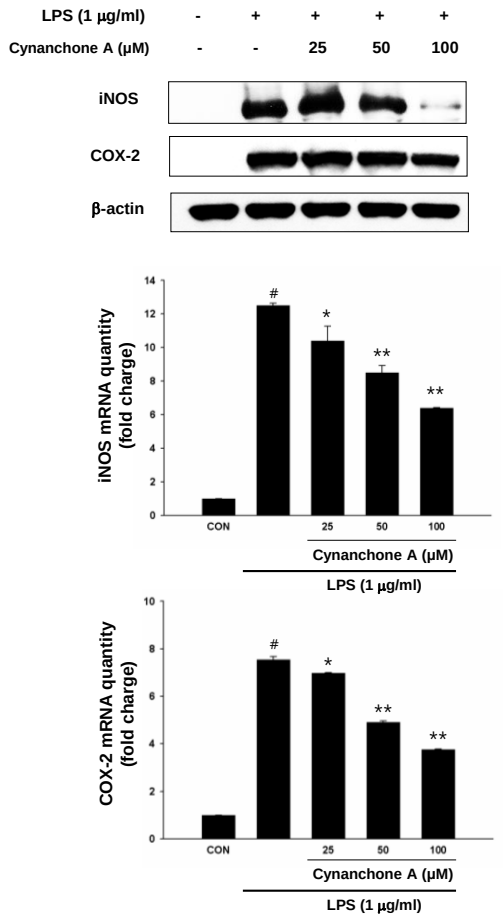


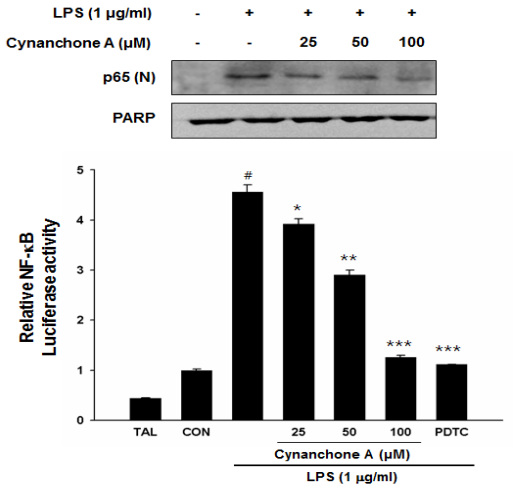
Figure 3. Inhibitory effects of cynanchone A on LPS-induced iNOS and COX-2 protein and mRNA expressions in RAW 264.7 cells. Each value indicates the mean ± SD and is representative of the results obtained from three independent experiments (#p<0.05 compared with the control; *p<0.05 and **p<0.01 compared with cells cultured with 1 μg/ml LPS).



3.3. Inhibitory effects of cynanchone a on LPS-induced NF-κB activation and on the nuclear translocations of p50 and p65

To investigate the mechanism responsible for the cynanchone A-mediated inhibitions of the transcriptions of iNOS, COX-2, and TNF-α, we investigated whether cynanchone A regulates LPS-induced NF-κB activity in LPS-stimulated RAW 264.7 macrophages using electrophoretic mobility shift assays (EMSA). As shown in <Figure 4A>, the DNA-binding activity of NF-κB was markedly increased by LPS alone (lane 4), whereas this binding was significantly reduced by cynanchone A pretreatment (lanes 5-7). The specific interaction between DNA and NF-κB was demonstrated by competitive inhibition using excess unlabelled NF-κB oligonucleotides (lane 2). In addition, we also investigated whether cynanchone A prevents the translocations of the p50 and p65 subunits of NF-κB to the nucleus using Western blotting. Negligible levels of nuclei p50 and p65 proteins were detected in control cells, whereas treatment with LPS for 1 h induced the nuclear translocation of both subunits. Western blotting analysis revealed that cynanchone A pretreatment dose-dependently attenuated p50 and p65 levels in nuclear fractions <Figure 4B>. These results suggest that cynanchone A inhibits NF-κB activation by preventing the LPS-induced nuclear translocations of both p50 and p65.

Figure 4. Inhibitory effect of cynanchone A on nuclear translocation of NF-κB. Each value indicates the mean ± SD and is representative of the results obtained from three independent experiments (#p<0.05 compared with the control; *p<0.05 and **p<0.01 compared with cells cultured with 1 μg/ml LPS).



4. Discussion

Cynanchone A is found in plants, such as, Ocotea suaveloens, Desfontainia spinosa, Hyptis capitata, and Vochysia divergens[15]. Zhang et al. found that low-density lipoprotein receptor-knockout mice fed a cholesterol-rich diet exhibited decreases in atherosclerotic lesion areas of over 50% when they were administered cynanchone A as compared with vehicle treated controls[16]. Fur-

thermore, cynanchone A was reported to suppress in vitro platelet aggregation, particularly when this was induced by epinephrine[17]. In addition, the anti-proliferative and anti-cancer activities of cynanchone A have been shown to be mediated by the inhibition of α and β DNA polymerases in human gastric cancer cells[12]. Moreover, in a soft agar colony model of TPA-induced skin cancer, cynanchone A treatment reduced tumor growth rates in JB6 cells[18]. Furthermore, previous reports indicate that cynanchone A is non-toxic to normal cells[12]. In murine macrophage RAW 264.7 cells, LPS induces the expression of iNOS, and thus, increases NO production. LPS stimulation is also known to induce I B proteolysis and NF-B nuclear translocation[19]. Therefore, RAW 264.7 cells provide us with an excellent model for drug screening and for subsequently evaluating potential inhibitors of the pathways leading to the induction of iNOS and the production of NO, a major macrophage-derived inflammatory mediator, which has also been reported to be involved in the pathogenesis of many inflammatory diseases[20]. It is well known that macrophages play a crucial role in both non-specific and acquired immune responses. For example, macrophage activation by LPS leads to a functionally diverse series of responses, which include the synthesis and production of NO, prostanoids, and proinflammatory cytokines. In the present study, we evaluated the effects of cynanchone A on the expressions and productions of several pro-inflammatory mediators (iNOS, COX-2, TNF-α) in LPS-activated macrophages, and found that LPS-induced NO, PGE_2, and TNF-α productions were inhibited dose-dependently by cynanchone A. To explore further the mechanisms underlying these inhibitions, we examined the expressions of iNOS and COX-2 proteins and mRNAs. It was found that cynanchone A inhibited the expressions of iNOS and COX-2 mRNA simultaneously and concentration-dependently <Figure 3>, suggesting that the inhibition of NO and PGE2 release might be attributable to the suppressions of iNOS and COX-2 expression at the mRNA level. It has been reported that TNF-α functions as a pro-inflammatory cytokine in vitro and in vivo[21], and that its production is crucially required for NO synthesis in IFN-γ and/or LPS-stimulated macrophages[22]. Furthermore, TNF-α elicits a number of physiological activities including septic shock, inflammation, cachexia, and cytotoxicity[23]. In the present study, cynanchone A was found to inhibit the expression of TNF-α mRNA <Figure 4>. Of the several transcription factors activated by inflammatory stimuli, NF-κB is known to play critical roles in the expression of pro-inflammatory enzymes and cytokines, such as, iNOS, COX-2, and TNF-α[24]. In the present study, we examined the possibility that cynanchone A inhibits NF-κB activity in vitro, and found that cynanchone A effectively prevented LPS-inducible NF-κB-DNA binding activity. In addition, we found that cynanchone A dose-dependently inhibited the translocation of activated NF-κB to the nucleus and the degradation and phosphorylation of IκB-α.

5. Conclusion

In conclusion, our results demonstrate that cynanchone A exerts anti-inflammatory effects by inhibiting NF-κB activation in macrophages, and thus, prevents the expressions of iNOS, COX-2, and TNF-α. Accordingly, our results suggest that cynanchone A may have a potential agent for inflammatory disease.

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