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Polyacetylene Compound from *Cirsium japonicum* var. *ussuriense* Inhibits the LPS-Induced Inflammatory Reaction via Suppression of NF-κB Activity in RAW 264.7 Cells

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Abstract

Cirsium japonicum var. *ussuriense* is known to have a variety of biological activities, including anti-inflammatory, analgesic activity and antipyretic activity. In this study we investigated the role of polyacetylene compound, 1-Heptadecene-11, 13-diyne-8, 9, 10-triol (PA) from the root of *Cirsium japonicum var. ussuriense* as an immune-modulator. PA was evaluated as inhibitors of some macrophage functions involved in the inflammatory process. We tested the effect of PA on the production of pro-inflammatory cytokines, interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), and nitric oxide (NO) in murine macrophage cell line, RAW264.7. There was no effect on cytokine production of macrophages by PA itself. However, PA inhibited lipopolysaccharide (LPS)-induced IL-1β and TNF-α production by macrophages at a dose dependent manner. PA also suppressed the NO production of macrophages by LPS. LPS-induced NF-κB activity was decreased by treatment of PA. Therefore, these results suggest that PA has anti-inflammatory effect by inhibiting the NF-κB activation.

Key Words: Polyacetylene compound, *Cirsium japonicum var. ussuriense*, Anti-inflammatory activity, RAW264.7 cell

INTRODUCTION

Inflammation is a complex and an essential protective response by the host immune system against physical, chemical and infective agents. However, it is frequent that inflammatory response to several stimuli leads to the damaging of normal tissues (Nathan, 2002; Rankin, 2004; Cho *et al.*, 2008).

Macrophages are innate immune cells and activation of macrophages plays an important role in the processes of several inflammatory diseases. Activated macrophages can produce various pro-inflammatory cytokines which may exert inflammatory effects. Inhibition of macrophages activation may alleviate inflammation under these conditions. The major cytokines that mediate inflammation are IL-1β and TNF-α. IL-1β and TNF-α share many biologic properties. Nitric oxide (NO) also plays an important role in vascular function during inflammatory responses. Their secretion can be stimulated by lipopolysaccharide (LPS), immune complexes, toxins, physical injury, and a variety of inflammatory processes. Nuclear factor-kappa B (NF-κB) is an important transcription factor and regulates various cellular genes and protein involved in immune and inflammatory responses, such as iNOS, tumor necrosis factor (TNF)-α and interleukin (IL)-6.

Cirsium japonicum var. *ussuriense* belongs to the compositae family and is perennial plant, 0.5 -2.0 m in height, possessing lance-shaped, spiny-toothed leaves, and white to purple flowers. Most plants of the Compositae family have been traditionally used in oriental medicine as an analgesic, diuretic, anti phlogistics, hemostatic, and detoxifying agents (Ganzera *et al.*, 2005). Linarin, luteolin, acacetin etc. from the Compositae family has been reported to have various biological activities including anti-oxidant activity (Park *et al.*, 2004; Jeong *et al.*, 2008). However, no report has been issued on anti-inflammatory activity or mode of action by 1-Heptadecene-11, 13-diyne-8, 9, 10-triol (PA) from the root of *Cirsium japonicum* var. *ussuriense.*

To search for the novel therapeutic agents against inflammatory diseases, therefore, the present study was designed to explore the potential anti-inflammatory effects of PA from the root of *Cirsium japonicum* var. *ussuriense.* We tested the effect of PA on the production of pro-inflammatory cytokines, IL-1β and TNF-α, and NO in mouse macrophage cell line, RAW264.7.

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MATERIALS AND METHODS

Plant material, isolation and characterization of polyacetylene compound

The root of *Cirsium japonicum* var. *ussuriense* (Compositae), which was collected in Kyunggi Province Korea May, 2007 and identified by Prof. Sookyeon Lee, of Sahmyook University. The voucher specimen (SYU-2007-01) was deposited at the Herbarium of the Sahmyook University.

Isolation and preparation of polyacetylene compound

The air-dried plant material (root, 1 kg) was sliced and extracted with 70% hot MeOH three times for 3 hours each. The extract was filtered, and concentrated *in vacuo* to get a MeOH extract. The concentrated extract (200 g) was suspended in water and then partitioned with *n*-hexane successively by EtOAc and *n*-Butanol to afford three fractions and an aqueous residue. The BuOH extracts obtained by removal of the solvent. Polyacetylene compound (PA) was isolated from BuOH fraction by silica gel column chromatography with gradient elution with MeOH and $CHCl_3$. This compound was characterized by NMR (Bruker AVANCE 400 NMR spectrometer). The purified PA, 1-Heptadecene-11, 13-diyne-8, 9, 10-triol with a molecular weight of 278 (Fig. 1), was dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich Korea, Yongin-city, Kyunggi-do, Korea) as stock solution, used directly for cell culture treatment. The final concentration of DMSO in the culture media was 1.0%, which did not show any effect in the assay system.

Cell culture and treatments

Murine macrophage cell line, RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 20 mM HEPES, 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Sigma-Aldrich Korea). The RAW cells were plated onto 6-well plate at 2×10^6 cells per well and incubated overnight. The cells were pretreated with various concentration of PA for 1 h and then treated with LPS (100 ng/ml, Sigma-Aldrich Korea) from *E. coli* O111:B4 for 24 h.

Measurement of cell viability

Trypan blue (Sigma-Aldrich Korea) exclusion was used to determine viable and dead cells to test cytotoxicity of PA. MTT (Methylthiazolyldiphenyl-tetrazolium bromide, Sigma-Aldrich Korea) assay was also used to measure the cytotoxicity on the cells with PA.

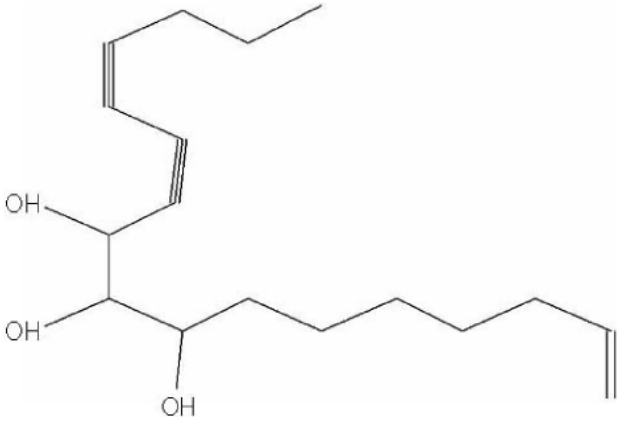

Fig. 1. The structure of polyacetylene compound (PA), 1-Heptadecene-11, 13-diyne-8, 9, 10-triol from the root of *Cirsium japonicum* var. *ussuriense*.

Cytokine assay

Cell culture supernatants were assayed for mouse IL-1β (eBioscience, San Diego, CA, USA) and TNF-α (Pharmingen, BD Biosciences, San Diego, CA, USA) by ELISA.

Nitric oxide determination

NO levels were measured with cell culture supernatants by Griess reaction. Briefly, 50 μl samples were incubated with 50 μl of 1% sulfanilamide (Sigma-Aldrich Korea) solution and 50 μl of 0.1% *N*-1-naphtylethylenediamine dihydrochloride (Sigma-Aldrich Korea) solution at room temperature for 10 min. The data was recorded and analyzed using SOFTmax version 4.6 software (Molecular Devices, Menlo Park, CA).

NF-κB activity

Cytosolic and nuclear extracts were isolated and assayed for NF-κB activity by colorimetric method system (NF-κB EZ-TFA Transcription Factor Assay, Upstate & Millipore, Billerica, MA, USA) according to the manufacturer's instruction.

Statistical analysis

All data and statistical analyses were carried out using SigmaStat, version 3.1, by one-way analysis of variance (ANOVA), and significance was accepted at $p<0.05$.

RESULTS

NMR characterization of polyacetylene compound (PA)

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.98, (*t*, H-17), 1.2-1.6 (H-4-7), 2.09 (*m*, H-3), 2.27 (*t*, *J*=7.1 Hz, H-15), 3.53 (*dd*, *J*=4.2 Hz, H-9), 4.04 (*td*, H-8), 4.58 (*d*, H-10), 5.01 (*d*, H-1), 5.26 (*dd*, H-9), 5.54 (*d*, H-10), 5.81 (*dt*, H-2), FABMS *m/z*: 301[M+Na^+], 279[M +H^+], 261, 243.

Cytotoxicity of PA to RAW 264.7 cells

The cytotoxic effect of PA was evaluated in RAW 264.7 cells using MTT assay and trypanblue exclusion method, but no effect on cell viability was observed at the concentrations used and at even higher dose, 400 μM, which showed the inhibitory effect on pro-inflammatory cytokines production (data not shown).

Inhibitory effect of PA on pro-inflammatory cytokines production

As a part of our screening study to evaluate the anti-inflammatory potentials of natural compounds for the development of new anti-inflammatory drug, we investigated the *in vitro* anti-inflammatory activity of the PA from the root of *Cirsium japonicum* var. *ussuriense*. It has been reported that cytokines, such as TNF-α and IL-1β, are pro-inflammatory mediators *in vitro* and *in vivo* (Feldmann *et al.*, 1991). To assess whether PA inhibits the pro-inflammatory mediators, we first investigated the effect of PA (50, 100, 200, and 400 μM) on LPS-induced TNF-α and IL-1β release in RAW 264.7 cells by ELISA. Our data showed that PA markedly suppressed LPS-induced IL-1β

and TNF-α production by macrophages at a dose dependent manner (Fig. 2).

Effect of PA inhibited nitric oxide (NO) production by LPS in RAW 264. 7 cells

It is marked by the accumulation of variety of inflammatory cells and release of several soluble mediators of inflammation such as reactive oxygen species (ROS), lipid mediators, proteases and cytokines (Huerre and Gounon, 1996; Rankin, 2004). Nitric oxides (NOs) are well known pro-inflammatory mediators (Vane *et al.*, 1994). Therefore, we next study the effect of PA on LPS-induced NO production in macrophages. To assess the effect of PA on LPS-induced NO production in RAW 264.7 macrophage cells, cells were treated with or without PA for 1 hr, and then with LPS for 24 hrs. Cell culture supernatants were collected and NO levels were determined using the Griess reaction (Fig. 3). LPS induced NO production 9-fold more than the control group, and PA inhibited this NO production in a dose-dependent manner in the range of 100-400 μM (Fig. 3).

The inhibitory effect of PA on LPS-induced NF-κB activation in macrophages

NF-κB is known to play a critical role in the regulation of genes and proteins involved in cell survival and inflammation. To explore the mechanism of the effects of PA on LPS-induced inflammation in RAW 264.7 macrophage cells, the protein levels of NO, TNF-α, and IL-1β were examined. PA suppressed the release of NO (Fig. 3) and proinflammatory cytokines, TNF-α and IL-1β proteins, in a dose-dependent manner (Fig. 2). Thus, we postulated that the inhibitory effect of PA on pro-inflammatory cytokines release may be associated with the

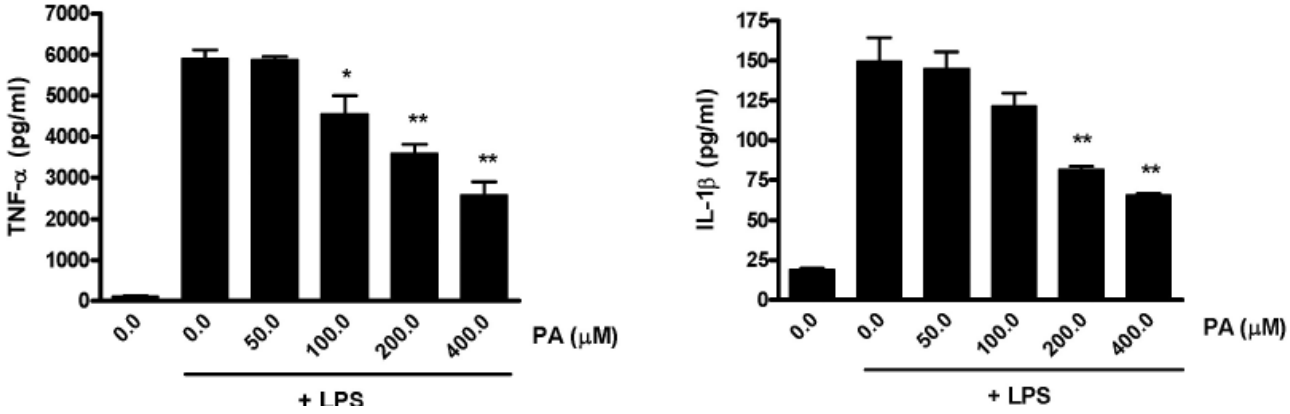

Fig. 2. The induction of IL-1β and TNF-α secretions by LPS were inhibited with treatment of PA at a dose dependent manner in RAW 264.7 cells. RAW264.7 cells were plated on 12 well cell culture plate, treated with or without PA at a dose dependent manner (50-400 μM) for 1 hr, and then with LPS (100 ng/ml). After 24 h of these treatments, cell culture supernatants were collected for cytokine (IL-1β and TNF-α) assay. Data are representative of at least three independent experiments, each done in triplicate; **p*<0.05, ***p*<0.01 compared to control groups treated with LPS alone.

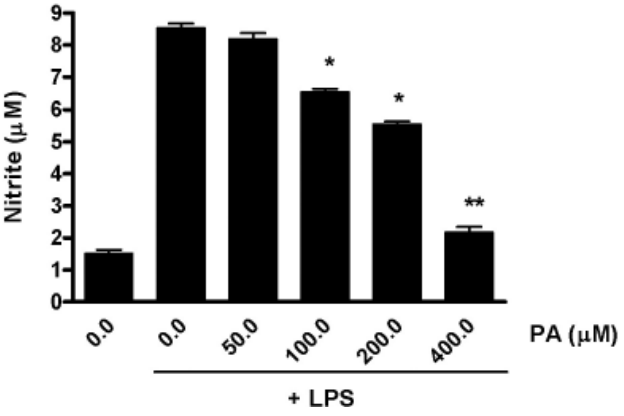

Fig. 3. The induction of nitric oxide by LPS was also inhibited with treatment of PA at a dose dependent manner in RAW 264.7 cells. RAW264.7 cells were treated with or without PA at a dose dependent manner for 1 hr, and then with LPS. After 24 h of these treatments, cell culture supernatants were collected for nitric oxide measurement. Data are representative of at least three independent experiments, each done in triplicate; **p*<0.05, ***p*<0.01 compared to control groups treated with LPS alone.

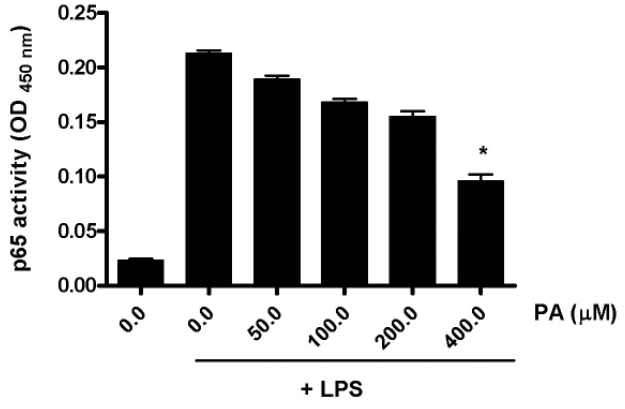

Fig. 4. The treatment of PA also decreased the NF-κB activity induced by LPS at a dose dependent manner. RAW264.7 cells were treated with or without PA at a dose dependent manner for 1 hr, and then with LPS. After 24 h of these treatments, Cytosolic and nuclear extracts were isolated and assayed for NF-κB activity by the colorimetric method. Data are representative of at least three independent experiments, each done in triplicate; **p*<0.05 compared to control groups treated with LPS alone.

transcriptional suppressions of NF-κB.

Since the activation of NF-κB is known to mediate LPS-induced expression of NO and TNF-α (Krasnow *et al.*, 1991; Doyle and O'Neill, 2006), colorimetric assay was performed to determine whether PA suppresses NF-κB activation. The result shows LPS-induced NF-κB activity was decreased by treatment of PA (Fig. 4).

DISCUSSION

In the absence of inflammation, wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism. However, chronic inflammation can also lead to some diseases, such as atherosclerosis and rheumatoid arthritis. It is for that reason that inflammation is normally regulated by the body. Despite of many efforts to develop anti-inflammatory agents, there is still a large demand for developing new agents (Cho *et al.*, 2008; Jin *et al.*, 2009). During our research for novel anti-inflammatory agents from natural products, we found that the PA of *Cirsium japonicum* var. *ussuriense* inhibits LPS-induced NO and pro-inflammatory cytokines production in a dose-dependent manner.

Macrophages play an important role in the initiation and amplification of a variety of inflammatory disease e.g. septic shock and chronic inflammatory conditions such as asthma, rheumatoid arthritis and Crohn's disease (Gordon, 2007). LPS stimulates toll-like receptor 4 (TLR4) on the surface of macrophages and triggers cytoplasmic adapter proteins, which results in the activation of NF-κB. The activated NF-κB is translocated into the nucleus where it induces the transcription of genes with an NF-κB consensus site in their promoter region, including iNOS and pro-inflammatory cytokines such as TNF-α, IL-1β. TNF-α elicits a number of physiological effects such as septic shock, inflammation, and cachexia (Aggarwal and Natarajan, 1996). IL-1β is a multifunctional cytokine that is responsible for mediating a variety of processes in the host defense response, inflammation, and the response to injury. Macrophages and many other cell types produce IL-1β by the actions of various stimuli such as viruses and LPS, the cell wall component from gram negative bacteria. Since IL-1β is a highly pro-inflammatory cytokines, agents that reduce its production and/or activity might be of a particular pharmacological and clinical interest.

As cytokines are critical to the pathogenesis of inflammatory disorders, inhibition of their production provides therapeutic targets in various inflammatory diseases. In the present study, we have demonstrated that PA significantly inhibits LPS-induced transcriptional activity of NF-κB, consequently resulting in a decrease in the expression of NO and pro-inflammatory cytokines, TNF-α and IL-1β, in RAW 264.7 cells. Therefore, inhibition of the pro-inflammatory cytokines production (Kim *et al.*, 2007) is believed to be one of the pathways by which PA has its anti-inflammatory activity in this study. ASA (Acetyl salicylic acid) was used as a positive control (data not shown). Whereas ASA has EC 50 at concentration of 4 mM, EC50 of PA is at concentration of above 200 μM.

Caspase-1 is a cysteine protease originally cloned as IL-1β-converting enzyme (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992). It is an essential regulator of inflammatory responses through its capacity to process and activate proIL-1β and proIL-18 (Ogura *et al.*, 2006). Considering that the release of IL-1β protein is dependent on the activation of caspase-1, further study is necessary to elucidate the exact mechanism of PA actions in terms of caspase-1 activation and its regulation.

In summary, these findings suggest that PA is a potent inhibitor of LPS-induced NO, TNF-α, and IL-1β production in RAW 264.7 macrophage cells. Moreover, these inhibitory effects of PA were found to be associated with NF-κB inactivation. Since NF-κB is a transcription factor that regulates the transcriptions of many inflammation-related genes, its inhibition by PA may offer a possible approach to the prevention or treatment of severe inflammatory diseases.

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