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Methyl jasmonate inhibits lipopolysaccharide-induced inflammatory cytokine production via mitogen-activated protein kinase and nuclear factor-κB pathways in RAW 264.7 cells

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Methyl jasmonate is an important signaling molecule involved in plant defense as well as in the regulation of plant growth and development. Despite its various functions in plants, its effects on animal cells have not been widely studied and no report has been issued on the molecular aspects of its anti-inflammatory effect. In the present study, we investigated the *in vitro* anti-inflammatory properties of methyl jasmonate in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Methyl jasmonate treatment effectively inhibited LPS-induced production of pro-inflammatory mediators (nitric oxide and prostaglandin E_2) and cytokines (tumor necrosis factor-α, interleukin (IL)-1β, and IL-6) in a concentration-dependent manner. Furthermore, it attenuated the LPS-induced activation of nuclear factor-κB (NF-κB) by suppressing the degradation of the inhibitor of κB-α (IκB-α). Additionally, methyl jasmonate dose-dependently blocked the phosphorylation of mitogen-activated protein kinases (MAPKs), i.e., p38 kinase, extracellular signal-regulated kinase (ERK) 1/2, and c-Jun N-terminal kinase (JNK), in these cells. These results suggest that methyl jasmonate attenuated the LPS-induced release of pro-inflammatory mediators and cytokines by suppressing the activation of MAPK (JNK, ERK and p38) and NF-κB signaling. This study not only demonstrated that methyl jasmonate exerts anti-inflammatory activities in macrophages but also revealed its potential as a candidate for the treatment of various inflammation-associated diseases.

1. Introduction

Inflammation is one of the body's self-defense mechanisms against pathogens and irritation. Under normal physiological conditions, inflammation protects our body from trauma, infection, and tissue injury. Macrophages and mast cells play important roles in the immune response against invading pathogens such as bacteria and viruses (Cotran et al. 1999; Chen et al. 2013). Macrophages respond to these threats by secreting inflammatory mediators including nitric oxide (NO), prostaglandin E_2 (PGE_2), and other pro-inflammatory cytokines that attract immune cells to the site of infection to eliminate the pathogens (Yang et al. 2013; Kim et al. 2014).

However, excessive chronic inflammation can cause serious damage to our body. For instance, overproduction of inflammatory mediators may lead to complications such as rheumatoid arthritis, atherosclerosis, asthma, and pulmonary fibrosis (Paul et al. 1999). Thus, regulating their production in tissues is one of the ways by which inflammation can be addressed.

Methyl jasmonate is a volatile organic compound involved primarily in plant defense, and regulates diverse plant developmental processes such as seed germination, root growth, flowering, fruit ripening, and senescence (Cheong and Choi 2003). It is derived from jasmonic acid through a reaction catalyzed by *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (Xu et al. 2003). Plants produce jasmonic acid and methyl jasmonate in response to biotic and abiotic stresses (particularly from herbivory and wounding) that build up in the damaged parts of the plant. Methyl jasmonate induces the synthesis of various defense chemicals such as phytoalexins (antimicrobial), nicotine, and proteinase inhibitors, which can affect flowering time, flower morphology, and the number of open flowers (Farmer and Ryan 1990; Radhika et al. 2010).

However, its effects on animal cells have not been demonstrated, and there are no reports on the molecular mechanism of its anti-inflammatory effect. Therefore, we investigated the anti-inflammatory properties of methyl jasmonate *in vitro* using lipopolysaccharide (LPS)-treated RAW 264.7 cells in order to elucidate its molecular action.

2. Investigations and results

2.1. Effects of methyl jasmonate on LPS-induced NO and PGE_2 production, and on cell viability

To investigate the effects of methyl jasmonate on LPS-induced NO and PGE_2 production in RAW 264.7 cells, the culture media were harvested, and nitrite and PGE_2 levels were measured using Griess reagent and enzyme-linked immunosorbent assay (ELISA), respectively. Cells treated with LPS alone showed markedly increased NO and PGE_2 production compared with normal cells. Addition of methyl jasmonate on the other hand, significantly inhibited both NO and PGE_2 production, with half-maximal inhibitory concentration (IC_{50}) values of 2.74 and 4.01 μM, respectively (Figs. 1, 2).

The cytotoxicity of the compound towards RAW 264.7 cells was evaluated by lactate dehydrogenase (LDH) assay. Methyl jasmonate concentrations (0.39–3.125 μM) used to inhibit NO and PGE_2 production had no negative effect on cell viability (Fig. 1).

2.2. Effects of methyl jasmonate on LPS-induced COX-2 protein expression

Since methyl jasmonate was found to inhibit PGE_2 production, we investigated whether this inhibitory effect was related to the regulation of cyclooxygenase (COX)-2 using western blot. As shown in Fig. 3, cells treated with LPS alone exhibited markedly higher COX-2 expression than normal cells, which was strongly inhibited

by methyl jasmonate in a concentration dependent manner. These results suggested that methyl jasmonate inhibits PGE_2 production through the downregulation of COX-2 expression.

2.3. Effects of methyl jasmonate on LPS-induced pro-inflammatory cytokine production

We further examined the inhibitory activities of methyl jasmonate against pro-inflammatory mediators by investigating its effects on the LPS-induced production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 by ELISA. As shown in Fig. 4, LPS-induced release of TNF-α was significantly inhibited by pretreatment with methyl jasmonate in a dose-dependent manner, with TNF-α production decreasing by about 54.6% at a methyl jasmonate concentration of 3.125 μM. Expressions of IL-6 and IL-1β were also similarly suppressed by methyl jasmonate (Figs. 5, 6).

2.4. Effects of methyl jasmonate on LPS-induced activation of MAPKs and NF-κB signaling in RAW 264.7 cells

Mitogen-activated protein kinases (MAPKs) play critical roles in the regulation of cell growth and differentiation, and control cellular responses to cytokines and stress (Guha and Mackman 2001). The three MAP kinases, c-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK) 1/2, are known to be involved in the LPS-induced pro-inflammatory cytokine production. Following LPS treatment of RAW 264.7 cells for 15 min, the phosphorylation levels of JNK, p38 MAPK, and ERK 1/2 increased significantly. Pretreatment of the cells with methyl jasmonate, however, inhibited the LPS-induced phosphorylation of these kinases in a dose-dependent manner. On the other hand, the amount of non-phosphorylated MAPKs was not affected by either LPS or methyl jasmonate treatment (Fig. 7).

Nuclear factor-κB (NF-κB) is another important molecular component of inflammation (Karin and Ben-Neriah 2000). Inhibitor of κB-α (IκB-α), one of most important inhibitors of the NF-κB complex, inactivates NF-κB by preventing its translocation from the cytoplasm to the nucleus (Karin and Ben-Neriah 2000; Li and Verma 2002). The degradation of IκB-α and the subsequent nuclear translocation of p65 (NF-κB complex subunit) are crucial to the activation of NF-κB by various stimuli (Lawrence et al. 2002). Thus, we investigated the degradation of IκB-α using western blotting to demonstrate the inhibitory effect of methyl jasmonate against LPS-induced NF-κB activation. Consistent with the above finding that methyl jasmonate blocks MAPK signaling, the LPS-induced IκB-α degradation was also similarly suppressed by the compound in a dose-dependent manner (Fig. 8).

3. Discussion

In this study, we demonstrated for the first time that methyl jasmonate regulates pro-inflammatory mediator and cytokine production in LPS-treated RAW 264.7 cells through MAPK and NF-κB signaling pathways.

Firstly, in order to assess the cytotoxicity of methyl jasmonate toward RAW 264.7 cells, we performed a LDH assay to determine the viability of these cells. The cells exhibited similar viability before and after exposure to methyl jasmonate at concentrations less than 3.125 μM for 24 h (Fig. 1). Thus, its cytotoxic effect against RAW 264.7 cells was negligible. Methyl jasmonate effectively inhibited the LPS-induced production of NO and PGE_2 in RAW 264.7 cells down to 56.5% and 62.2%, respectively, at a concentration of 3.125 μM (Figs. 1, 2). In order to clarify whether the inhibitory effect of methyl jasmonate on PGE_2 release could

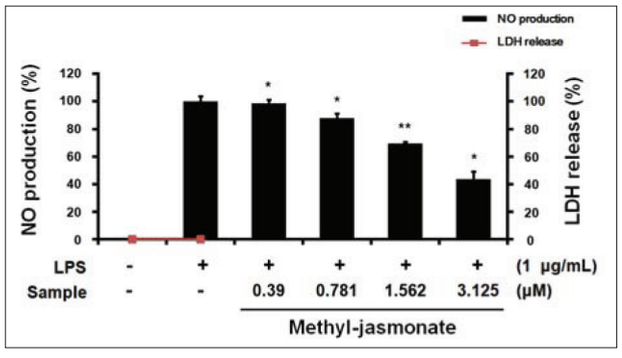

Fig. 1: Inhibitory effects of methyl jasmonate on NO production in RAW 264.7 cells. The production of NO was assayed in the culture medium of cells stimulated with LPS (1 μg/mL) for 24 h, in the presence of different concentrations (0.39, 0.781, 1.562, and 3.125 μM) of the compound. Cytotoxicity was determined by LDH assay. Data are expressed as the mean ± SEM of triplicate experiments. $*P < 0.05$; $**P < 0.01$.

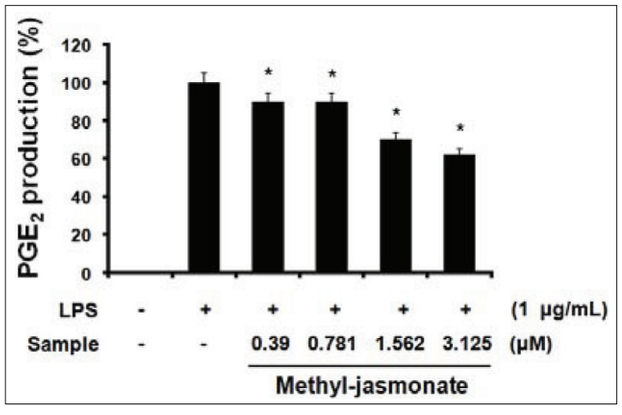

Fig. 2: Inhibitory effects of methyl jasmonate on PGE_2 production in RAW 264.7 cells. The production of PGE_2 was assayed in the culture medium of cells stimulated with LPS (1 μg/mL) for 24 h, in the presence of different concentrations (0.39, 0.781, 1.562, and 3.125 μM) of the compound. Data are expressed as the mean ± SEM of triplicate experiments. $*P < 0.05$; $**P < 0.01$.

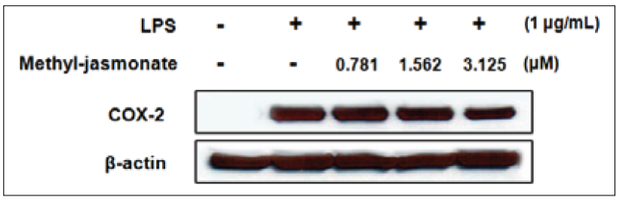

Fig. 3: Inhibitory effects of methyl jasmonate on the level of COX-2 protein expression in LPS-stimulated RAW 264.7 cells. Cells (1.0×10^6 cells/mL) were pre-incubated for 18 h, and then treated with LPS (1 μg/mL) and methyl jasmonate for 24 h. The protein levels were analyzed by western blot.

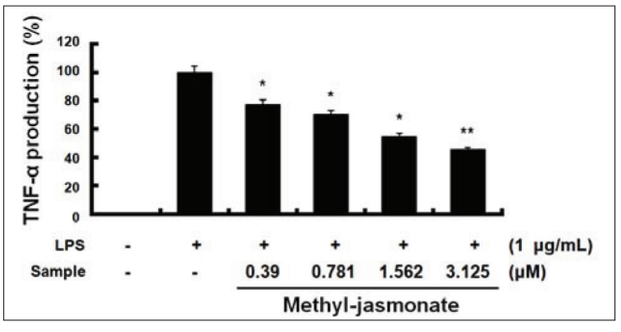

Fig. 4: Inhibitory effects of methyl jasmonate on TNF-α production in RAW 264.7 cells. The production of TNF-α was assayed in the culture medium of cells stimulated with LPS (1 μg/mL) for 24 h, in the presence of different concentrations (0.39, 0.781, 1.562, and 3.125 μM) of the compound. Data are expressed as the mean ± SEM of triplicate experiments. $*P < 0.05$; $**P < 0.01$.

result from decreased COX-2 expression, we examined its effect on COX-2 protein levels by western blot analysis. The expression of COX-2 was strongly induced by LPS, but was slightly suppressed in the presence of methyl jasmonate (Fig. 3).

Tumor necrosis factor-α is a particularly important component of the inflammatory response, and causes many of the clinical problems associated with acute inflammatory diseases (Simsek 2010). For example, the release of TNF-α and IL-1β is believed to play a considerable role in the pathophysiology of endometriosis (Ribeiro et al. 2000). Similarly, IL-6 is considered to be a pivotal pro-inflammatory cytokine and is regarded as an endogenous mediator of LPS-induced fever (Mihara et al. 2012). Hence, we examined the inhibitory effect of methyl jasmonate on TNF-α, IL-1β, and IL-6 production. The suppression of these cytokines by methyl jasmonate (Figs. 4–6) suggests that it possesses a great potential in treating inflammatory diseases.

The transcription factor NF-κB controls the expression of a number of genes such as inducible nitric oxide synthase (iNOS), COX-2, TNF-α, IL-1β, and IL-6 that are involved in immune response and inflammation. Inhibition of NF-κB activity by the overexpression of IκB-α has been reported to suppress inflammatory response (Karin and Ben-Neriah 2000; Li and Verma 2002; Lawrence et al. 2002). Similarly, we demonstrated that methyl jasmonate inhibited NF-κB activity in LPS-treated RAW 264.7 cells by preventing IκB-α degradation in a dose-dependent manner (Fig. 8). The activation of NF-κB is regulated by cellular kinases, including MAPKs, which are a highly conserved family of serine/threonine kinases that consists of JNK, ERK1/2, and p38 subgroups (Guha and Mackman 2001; Robinson and Cobb 1997). The inhibitory action of methyl jasmonate against MAPK activation was confirmed by the suppression of LPS-induced phosphorylation of these kinases upon pretreatment with the compound (Fig. 7).

In conclusion, these results suggest that methyl jasmonate attenuated the LPS-induced release of pro-inflammatory mediators and cytokines by suppressing the activation of MAPK (JNK, ERK, and p38) and NF-κB signaling. This study not only provides evidence that methyl jasmonate exerts anti-inflammatory activities in macrophages, but also sheds light on its potential use as a therapeutic agent for treating various inflammation-associated diseases.

4. Experimental

4.1. Cell culture

RAW 264.7 murine macrophages were purchased from the Korean Cell Line Bank (Seoul, Korea) and were maintained at sub-confluence in 5% CO_2 humidified atmosphere at 37 °C. The medium used for the routine subculture was Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL).

4.2. LDH assay for measuring cytotoxicity

RAW 264.7 cells (1.8×10^5 cells/mL) were plated in 24-well plates and pre-incubated for 18 h. They were then treated with the indicated concentrations of subtropical plant extracts for 2 h, before challenged with LPS (1 μg/mL) for an additional 18 h. The release of LDH from the cells was used to assess cytotoxicity using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA); LDH activity was determined from the production of nicotinamide adenine dinucleotide hydrate during the conversion of lactate to pyruvate. The optical density of the solution was measured at a wavelength of 490 nm.

4.3. Nitric oxide determination

Nitrite concentration in the medium was measured as an indicator of nitric oxide production according to the Griess reaction method. In brief, RAW 264.7 cells ($1.8 \times$

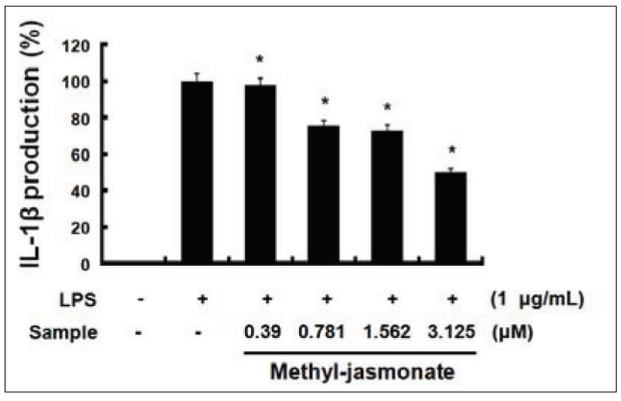

Fig. 5: Inhibitory effects of methyl jasmonate on IL-1β production in RAW 264.7 cells. The production of IL-1β was assayed in the culture medium of cells stimulated with LPS (1 μg/mL) for 24 h in the presence of different concentrations (0.39, 0.781, 1.562, and 3.125 μM) of the compound. Data are expressed as the mean ± SEM of triplicate experiments. $^*P < 0.05$; $^{**}P < 0.01$.

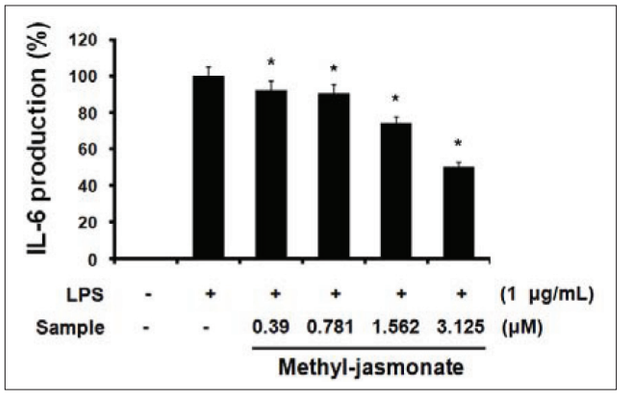

Fig. 6: Inhibitory effects of methyl jasmonate on IL-6 production in RAW 264.7 cells. The production of IL-6 was assayed in the culture medium of cells stimulated with LPS (1 μg/mL) for 24 h, in the presence of different concentrations (0.39, 0.781, 1.562, and 3.125 μM) of the compound. Data are expressed as the mean ± SEM of triplicate experiments. $^*P < 0.05$; $^{**}P < 0.01$.

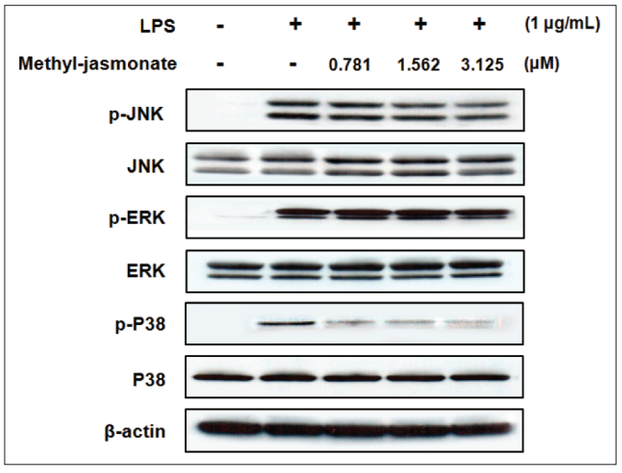

Fig. 7: Inhibitory effects of methyl jasmonate on the level of MAPK protein expression in LPS-stimulated RAW 264.7 cells. Cells (3.0×10^6 cells/mL) were pre-incubated for 18 h, and then treated with LPS (1 μg/mL) and methyl jasmonate for 15 min. The protein levels were determined via western blotting.

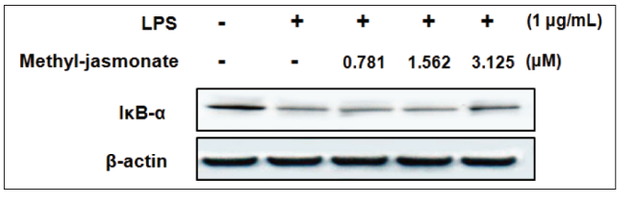

Fig. 8: Inhibitory effects of methyl jasmonate on the level of IκB-α protein expression in LPS-stimulated RAW 264.7 cells. Cells (3.0×10^6 cells/mL) were pre-incubated for 18 h, and then treated with LPS (1 μg/mL) and methyl jasmonate for 15 min. The protein levels were determined via western blotting.

10^5 cells/mL) were plated in 24-well plates, incubated for 24 h, and pretreated with the indicated concentrations of subtropical plant extracts for 2 h, before challenged with LPS (1 µg/mL) for an additional 18 h. Equal volumes of cultured medium and Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid) (Snell et al. 1996; Woo et al. 2005) were mixed at room temperature for 10 min, and the absorbance was measured at 540 nm.

4.4. Measurement of pro-inflammatory mediator (PGE2, IL-6, IL-1β, and TNF-α) levels

RAW 264.7 cells (1.8×10^5 cells/mL) were cultured in 24-well plates in the presence of various concentrations of extract samples and LPS (1 µg/mL), and incubated for 24 h. The cell culture medium was centrifuged at 13,000 rpm for 10 min, and the supernatant was collected for PGE_2, IL-6, IL-1β, and TNF-α analysis by ELISA, according to the manufacturer's instructions (mouse IL-6 and TNF-α ELISA: Invitrogen Inc., Carlsbad, CA, USA; mouse PGE_2 and IL-1β ELISA: Abcam Inc., Cambridge, UK).

4.5. Western blot analysis

After LPS stimulation for 24 h, RAW 264.7 cells were washed twice with cold phosphate-buffered saline. The cells were allowed to lyse in a lysis buffer (RIPA buffer, 1% Nonidet P-40, 1% protease inhibitor cocktail) for 1 h, before collected in microtubes and centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant was transferred into new microtubes, and the protein content of the cell lysates was determined with Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as standard. After heating at 70 °C for 10 min, equal amounts of the cell lysates were separated in a 4–12% Bis-Tris mini gel electrophoresis (Invitrogen Inc.) and transferred to nitrocellulose membrane (Invitrogen Inc.). The membrane was then washed with Tris-buffered saline (TBS, 20 mM Tris base, 137 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TTBS), and blocked with TTBS containing 5% BSA solution for 2 h. The membrane was incubated overnight with primary antibodies diluted in TTBS (1:1000) at 4 °C, and washed four times with TTBS. Primary antibodies (p-JNK, JNK, p-ERK, ERK, p-p38, p38, and IκB-α) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Each membrane was incubated for 1 h with secondary peroxidase-conjugated goat immunoglobulin G (IgG, 1:5000), and washed five times with TTBS. The target proteins were detected using an enhanced chemiluminescence solution. The immunoreactive bands were detected and exposed to X-ray film, and protein levels were quantified by scanning the immunoblots.

4.6. Data analysis

All data are expressed as the mean ± SEM of at least three replicates. Student's *t*-tests and one-way analysis of variance (ANOVA) were used for statistical analyses. Differences with *P* values of less than 0.05 were considered significant.

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Conflicts of interest: None declared.

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