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Anti-Inflammatory Effect of *Asterias amurensis* Fatty Acids through NF-κB and MAPK Pathways against LPS-Stimulated RAW264.7 Cells

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Asterias amurensis (starfish) is a marine organism that is harmful to the fishing industry, but is also a potential source of functional materials. The present study was conducted to analyze the profiles of fatty acids extracted from *A. amurensis* tissues and their anti-inflammatory effects on RAW264.7 macrophage cells. In different tissues, the component ratios of saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids differed; particularly, polyunsaturated fatty acids such as dihomo-gamma-linolenic acid (20:3n-6) and eicosapentaenoic acid (20:5n-3) were considerably different. In lipopolysaccharide-stimulated RAW264.7 cells, fatty acids from *A. amurensis* skin, gonads, and digestive glands exhibited anti-inflammatory activities by reducing nitric oxide production and inducing nitric oxide synthase gene expression. *Asterias amurensis* fatty acids effectively suppressed the expression of inflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and interleukin-6 in lipopolysaccharide-stimulated cells. Cyclooxygenase-2 and prostaglandin E_2, which are critical inflammation biomarkers, were also significantly suppressed. Furthermore, *A. amurensis* fatty acids reduced the phosphorylation of nuclear factor-κB p-65, p38, extracellular signal-related kinase 1/2, and c-Jun N-terminal kinase, indicating that these fatty acids ameliorated inflammation through the nuclear factor-κB and mitogen-activated protein kinase pathways. These results provide insight into the anti-inflammatory mechanism of *A. amurensis* fatty acids on immune cells and suggest that the species is a potential source of anti-inflammatory molecules.

Keywords: *Asterias amurensis*, fatty acids, anti-inflammation, nuclear factor-κB pathway, mitogen-activated protein kinase pathway

Introduction

Lipids containing fat-soluble vitamins and essential fatty acids are important for human health and development and play an important role in preventing diseases through changes in their compositions [1]. Particularly, metabolic changes in saturated fatty acids (SFAs) and their metabolic derivatives lead to many diseases such as cardiovascular disease and cancers [2, 3]. Monounsaturated fatty acids (MUFAs) such as palmitoleic acid (16:1) were reported to have beneficial effects on coronary heart disease [4], nonalcoholic fatty liver disease [5], and cardiovascular disease [6]; additionally, polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are significantly associated with fatty liver disease [7], autoimmune responses [8], and many chronic diseases including cardiovascular disease [9], cancers [10] and diabetes [11].

Particularly, omega-3 and omega-6 PUFAs containing more than 20 carbons are converted to diverse lipid mediators such as eicosanoids and docosanoids, which are prostaglandins, prostacyclins, and leukotrienes, by

cyclooxygenases or lipoxygenases [12, 13]. These molecules are critical cellular signaling components associated with inflammation and immune modulation in physiology [14]. Arachidonic acid (20:4n-6) is known as a pro-inflammatory eicosanoid, while EPA and dihomo-gamma-linolenic acid (DGLA, 20:3n-6) are precursors of anti-inflammatory eicosanoids [14, 15]. Moreover, DHA is a precursor of anti-inflammatory docosanoids such as resolvins and protectins [16], which are anti-lipid mediators used in cancer therapy [17]. Additionally, omega-3 PUFAs involving EPA and DHA were reported to have beneficial effects on immune-related diseases such as arthritis and asthma via positive immune regulation [14, 15].

The starfish *Asterias amurensis*, which is extensively distributed in the North Pacific Ocean and near the east coast of Korea, is a harmful organism that causes damage to the fishing industry [18]. However, starfish including *A. amurensis* have been investigated as potential sources of bioactive marine natural products. Various metabolites including steroids, saponins, steroidal glycosides, anthraquinones, alkaloids, phospholipids, and peptides were detected in starfish [19]. These compounds with unique structures are known to possess anti-tumor, anti-inflammatory [20], immunomodulation, anti-allergy, anti-fungal, hemolytic [21], antimutagenic [22], neuritogenic [23], cytotoxic, and anti-viral [24] activities. Particularly, *A. amurensis* was reported to be rich in EPA-bound phosphartidylcholine [25] and glycosylceramide [18]. However, no studies have examined the fatty acid profiles of starfish organs and immune modulation functions of these fatty acids against cytokines and associated signaling proteins in macrophage cells. Therefore, the current study was conducted to investigate the fatty acid profiles of *A. amurensis* organs and immune-regulation by fatty acids extracted from *A. amurensis* organs against the lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages.

Materials and Methods

Starfish Sample

Starfish samples were obtained from the East Sea near Gangwon Province, South Korea, in April 2015. The organs were divided into 3 groups, the skin, gonads, and digestive glands (d-gland), and subjected to fatty acid extraction.

Fatty Acid Extraction and Analysis

Fatty acid extraction was performed in quintuplicate ($n = 5$) from starfish organs as described by Garces and Mancha [26]. Fatty acid methyl esters were prepared in a one-step hydrolysis, extraction, and methylation method as described previously [27]. Prepared fatty acid methyl esters from each organ were analyzed by gas chromatography (GC)-flame ionization detection (Perkin Elmer, USA). For GC analysis, a capillary column was used (SPTM-2560, 100 m 0.25 mm i.d., film thickness 0.20 μm, Merck, Germany). The GC standards are shown in Table 2.

Macrophage Proliferation

RAW264.7 cells in RPMI-1640 medium (supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) were seeded into a 96-well plate at 1 10^5 cells/well. After incubation for 24 h, different concentrations of fatty acids (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, or 4.0%) were added and the plate was incubated for another 24 h. The experiment was performed in triplicate. The supernatants were discarded and an EZ-Cytox Cell Viability Assay Kit (DaeilLab Service, Korea) was used to evaluate the proliferation of macrophage cells as described by Kim *et al.* [28]. The macrophage proliferation ratio (%) was calculated based on the following formula.

Table 1. Sequences of oligonucleotide primers used for immune-associated genes in macrophage test.

Gene	Accession No.	Sequence	Product size (bp)
iNOS	BC062378.1	F: TTCCAGAATCCCTGGACAAG R: TGGTCAAACTCTTGGGGTTC	180
IL-1β	NM_008361.4	F: GGGCCTCAAAGGAAAGAATC R: TACCAGTTGGGGAACTCTGC	183
IL-6	NM_031168.2	F: AGTTGCCTTCTTGGGACTGA R: CAGAATTGCCATTGCACAAC	191
COX-2	NM_011198.4	F: AGAAGGAAATGGCTGCAGAA R: GCTCGGCTTCCAGTATTGAG	194
TNF-α	D84199.2	F: ATGAGCACAGAAAGCATGATC R: TACAGGCTTGTCACTCGAATT	276
β-Actin	NM_007393.5	F: CCACAGCTGAGAGGAAATC R: AAGGAAGGCTGGAAAAGAGC	193

$$\text{Macrophage proliferation ratio (\%)} = \frac{\text{the absorbance of the test group}}{\text{the absorbance of the control group}} \times 100$$

Nitric Oxide (NO) Production

The immunomodulatory activity of fatty acids was determined by measuring NO production in the macrophage culture supernatant. The cells were cultured with different concentrations of fatty acids and stimulated with 1 μg/ml of LPS. The NO concentration was evaluated using Griess reagent (Sigma-Aldrich, USA) [29, 30].

RNA Isolation and Reverse Transcription

Total RNA was isolated from macrophage RAW264.7 cells using Tri reagent (Molecular Research Center, Inc., USA). RNA was precipitated using 100% isopropanol and washed with 75% ethanol. The RNA pellet was dissolved in nuclease-free water and the concentration of the extracted RNA was measured using a nanophotometer (Implen, Germany). cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions.

Analysis of Immune Gene Expression by Quantitative Real-Time PCR

RAW264.7 immune mRNA was quantified using the QuantStudio 7 FlexReal-Time PCR System (Thermo Fisher Scientific, USA) in a 96-well format in a total reaction volume of 20 μl/well with SYBR Premix Ex Taq II (Takara Bio, Inc., Japan). The reaction mixture consisted of 0.4 μM of each specific primer pair (Table 1) and 0.1 ng of cDNA template. PCR amplification conditions were as follows: initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing at 55°C for 5 sec. The results of quantification were calculated using the $2^{-\Delta\Delta C}{}_T$ method [31] with comparison to β-actin as the reference mRNA.

Western Blot Assay

RAW264.7 cells were cultured with different concentrations of *A. amurensis* fatty acids followed by stimulation with 1 μg/ml of LPS. After 24 h of incubation, the cells were harvested with RIPA buffer (Tech & Innovation, Korea). The protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein (30 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Western blot analysis was carried out as described by Narayanan *et al*. [32]. The membranes were incubated with antibodies specific for p-NF-κB p65 (R&D Systems, USA), IκBα (Cell Signaling Technology, USA), p-p38 (R&D Systems), p-ERK1/2 (R&D Systems), p-JNK (R&D Systems), and β-actin (Santa Cruz Biotechnology, Inc., USA). Protein signals were detected using Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific).

Quantification of PGE_2

The concentration of prostaglandin E_2 (PGE_2) was determined using a PGE_2 ELISA Kit (Enzo Life Sciences, USA) according to the manufacturer's instructions. Color was developed using *p*-nitrophenyl phosphate substrate, and the optical density was evaluated at 405 nm. The experiment was performed in duplicate. The concentration of PGE_2 in the samples was calculated based on a standard curve.

Statistical Analysis

Statistical analysis was performed with 'Statistix 8.1' Statistics Software. Fatty acid analysis was conducted in triplicate, and the data are presented as the mean value with standard deviation.

Table 2. Fatty acid retention times.

Peak No.	Retention time (min)	Fatty acid
1	10.397	C4:0
2	11.082	C6:0
3	12.281	C8:0
4	14.251	C10:0
5	15.608	C11:0
6	17.058	C12:0
7	18.753	C13:0
8	20.430	C14:0
9	21.869	C14:1
10	23.605	C16:0
11	26.747	C17:1
12	27.349	C18:0
13	27.934	C18:1n9(t)
14	28.199	C18:1n9(c)
15	28.946	C19:0
16	29.604	C18:2n6(c)
17	30.528	C20:0
18	30.622	C18:3n6
19	31.237	C20:1
20	31.306	C18:3n3
21	32.093	C21:0
22	32.646	C20:2
23	33.645	C20:3n6
24	34.262	C22:1n9
25	34.395	C20:3n3
26	34.470	C20:4n6
27	35.211	C23:0
28	35.753	C22:2
29	36.341	C24:0
30	36.845	EPA
31	37.688	C24:1
32	41.116	DHA

Statistical differences were tested using one-way analysis of variance and Duncan's multiple-range test at $p < 0.05$. Macrophage proliferation and NO production results were compared by one-way analysis of variance followed by comparison with a model control and differences were considered significant at $p < 0.01$ and $p < 0.001$.

Results

Fatty Acid Analysis of Skin, Gonad, and d-Gland from *A. amurensis*

Total fatty acids from the skin, gonad, and d-gland of starfish samples are shown in Fig. 1. The fatty acids in each organ were divided into 3 groups, saturated, monounsaturated, and polyunsaturated fatty acids. The significant difference between the amounts of fatty acid in the same sample was indicated with the letters a-e. SFA from the skin accounted for 31.6 ± 1.8% of the total fatty acids, while unsaturated fatty acids including MUFA and PUFA showed values of 41.2 ± 0.7% and 27.3 ± 1.7%, respectively. Similarly, SFA from the gonad accounted for 27.6 ± 2.2% of the total fatty acid content and MUFA and PUFA were 40.3 ± 4.6% and 32.1 ± 2.7%; SFA from the d-gland was 27.4 ± 2.9% of the total fatty acids and MUFA and PUFA were 24.2 ± 3.5% and 48.2 ± 6.0%, respectively.

In most tissues, palmitic acid (16:0) and stearic acid (18:0) levels were the highest and SFA levels were similar to those in mammals such as humans and mice. The significant difference between the amount of same fatty acid in the

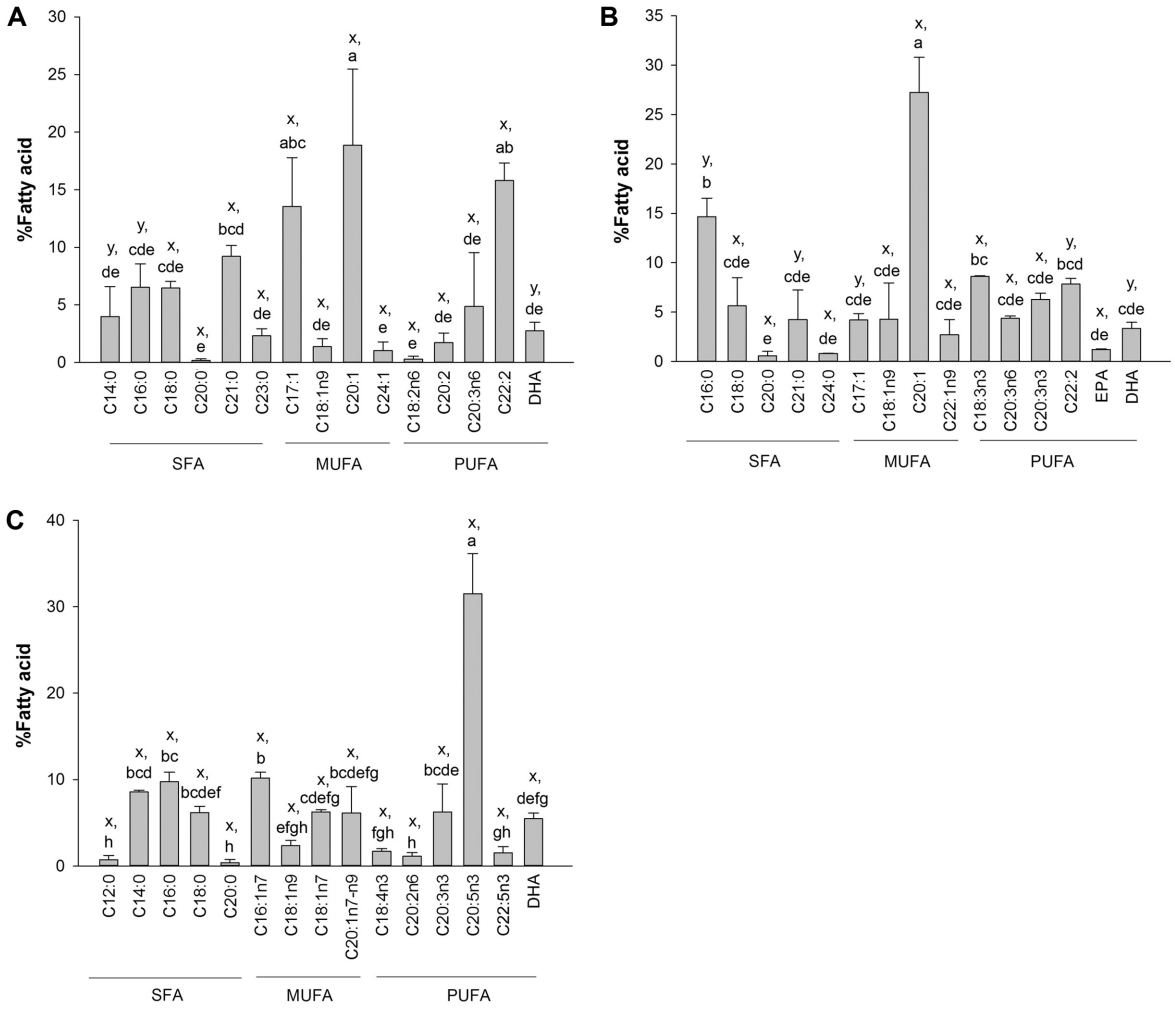

Fig. 1. Fatty acid composition of *A. amurensis* fatty acids.
(**A**) Skin fatty acid, (**B**) Gonad fatty acid, and (**C**) D-gland fatty acid. Data are presented as the means ± standard deviation ($n = 5$). The letters a–e showed a significant difference ($p < 0.05$) between the amount of fatty acid in the same sample, while x–z indicate a significant difference ($p < 0.05$) between the sources of each fatty acid. (SFA = Saturated fatty acid, MUFA = Monounsaturated fatty acid, and PUFA = Polyunsaturated fatty acid).

different sources was indicated with the letters x-z, whereas x > y > z. Among MUFAs, paullinic acid (20:1) showed high levels in the skin and gonad; however, palmitoleic acid (16:1n-7) and oleic acid (18:1) were higher than paullinic acid in the d-gland. PUFA considerably differed depending on the tissues; skin contained high omega-6 PUFAs such as dihomo-gamma-linolenic acid (DGLA, 20:3n-6) rather than omega-3 PUFA, whereas the gonad and d-gland contained much higher omega-3 PUFAs than omega-6 PUFAs. The gonad contained higher DHA than EPA levels, but the d-gland showed the highest EPA levels among the three tissues.

Effects of *A. amurensis* Fatty Acid on RAW264.7 Cell Proliferation

To determine whether fatty acids from different organs of starfish are toxic to RAW264.7, cell viability was tested with various concentrations of fatty acids using an EZ-Cytox Cell Viability Assay Kit (Fig. 2A). At up to a 2.0% fatty acid concentration, no cytotoxicity was observed in the three organs; additionally, skin fatty acids showed no cytotoxicity at any concentration. However, the gonad and d-gland showed low cell viability at concentrations higher than 3.0% and 2.5%, respectively, unlike skin fatty acids.

Asterias amurensis Fatty Acids Inhibited NO Production of LPS-Stimulated RAW264.7 Cells

NO plays an important role in pathophysiological conditions such as inflammation and infection [33]. To analyze the potential anti-inflammatory properties of fatty acids, RAW264.7 murine macrophage cells, which can produce NO, were cultured (Fig. 2B) and LPS was used to stimulate inflammation. Fatty acids extracted from three tissues significantly inhibited the production of LPS-stimulated NO according to the fatty acid concentration. Most fatty acid concentrations significantly inhibited NO production. However, the highest concentration of skin fatty acids (4.0%) showed the greatest inhibitory effect on NO production with values similar to those in the unstimulated group.

Asterias amurensis Fatty Acids Inhibited Immune-Associated Gene Expression of LPS-Stimulated RAW264.7 Cells

In LPS-stimulated RAW 264.7 cells, the expression levels of most immune-associated genes were dose-dependently inhibited depending on the concentration of *A. amurensis* fatty acids. For the three tissue fatty acids, gene expression slightly differed. Interleukin (IL)-1β expression was highly reduced by *A. amurensis* fatty acids, while the expression levels of other cytokine genes such as IL-6 and tumor

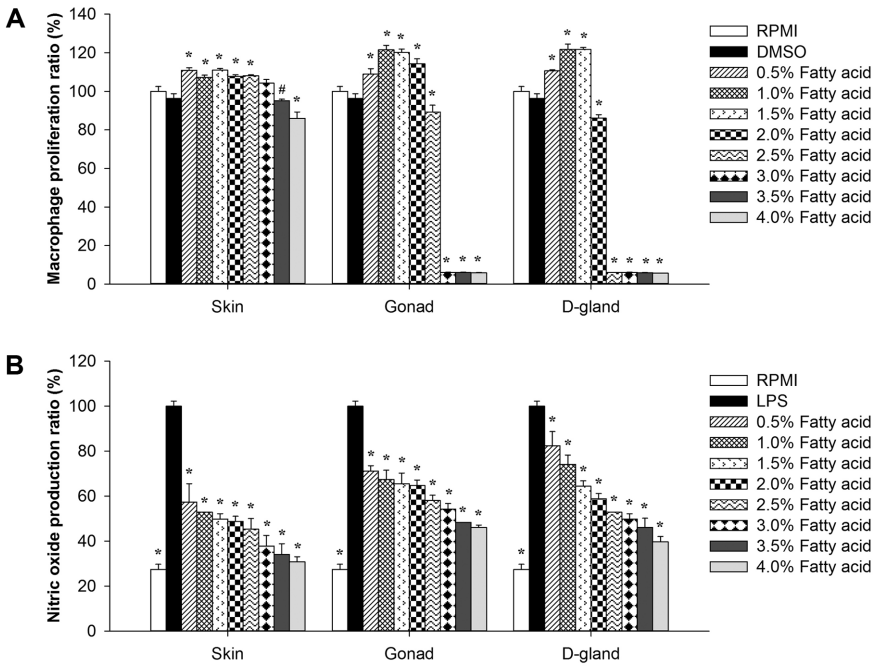

Fig. 2. Effects of fatty acid from different organs of *Asterias amurensis*, (**A**) Effect of *A. amurensis* fatty acids on macrophage proliferation and (**B**) effect of *A. amurensis* fatty acids on nitric oxide production under LPS-stimulated conditions (#$p < 0.01$ and *$p < 0.001$).

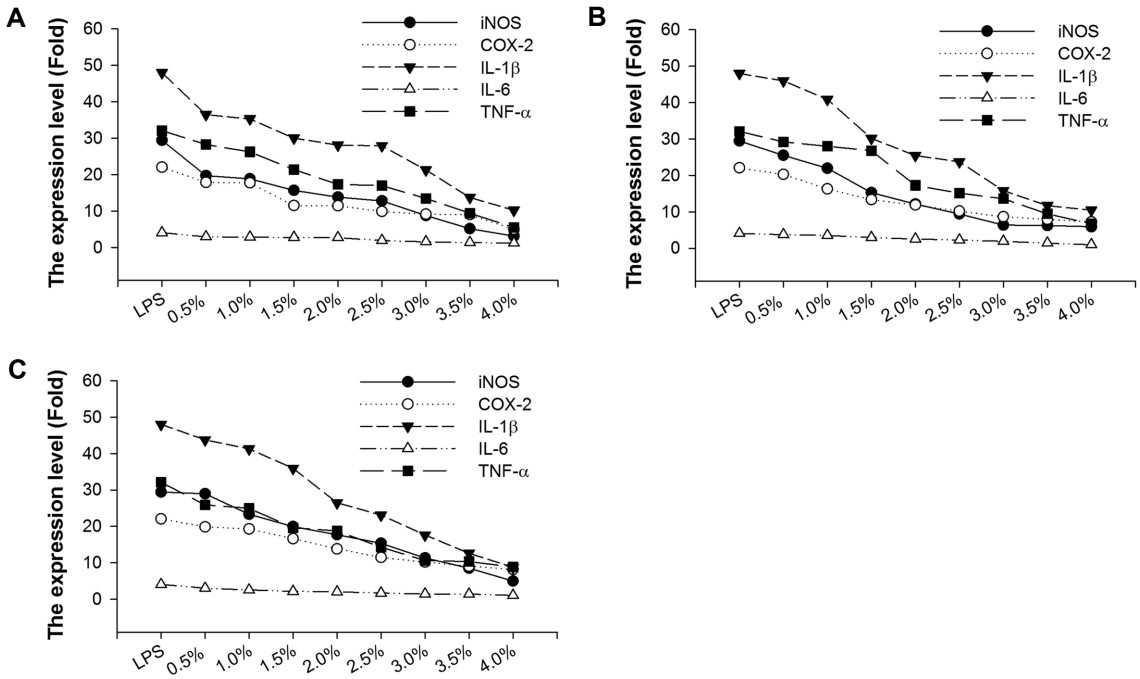

Fig. 3. Relative expression (fold-change) of immune-related genes from LPS-stimulated RAW264.7 cells; relative expression of (**A**) skin fatty acid samples, (**B**) gonad fatty acid samples, and (**C**) d-gland fatty acid samples.

necrosis factor (TNF)-α were dose-dependently decreased. Moreover, the expression of inducible NO synthase (iNOS), a key enzyme which generates NO, was reduced in a dose-dependent manner following treatment with skin, gonad, and d-gland fatty acids (Figs. 3A–3C), which parallels the results for NO production. LPS-stimulated RAW 264.7 cells showed inflammation and up-regulation of cyclooxygenase (COX)-2, another well-known inflammatory biomarker [34]. In contrast, the expression levels of COX-2 were dose-dependently down-regulated according to the concentration of *A. amurensis* fatty acids.

Asterias amurensis Fatty Acids Inhibited PGE_2 Production of LPS-Stimulated RAW264.7 Cells

The anti-inflammatory effects of *A. amurensis* fatty acids on RAW264.7 cells were evaluated by measuring the level of PGE_2, a critical immune biomarker with pro-inflammatory biological activity, after LPS-induced inflammation. Fig. 4 shows that the production of PGE_2 was dose-dependently inhibited with increasing concentrations of *A. amurensis* fatty acids in LPS-stimulated RAW 264.7 cells. The results showed that PGE_2 levels were dramatically decreased in the skin, gonad, and d-gland fatty acids by comparison with cells treated with LPS alone. Among the tissue fatty acids, skin fatty acids had greater effects on the PGE_2 levels in cells when compared to those from the other two tissues.

Asterias amurensis Fatty Acids Inhibited Inflammation through the MAPK and NF-κB Signaling Pathways in LPS-Stimulated RAW264.7 Cells

To determine whether *A. amurensis* fatty acids have anti-inflammatory effects in RAW264.7 cells, activation of immune-related molecules such as nuclear factor (NF)-κB

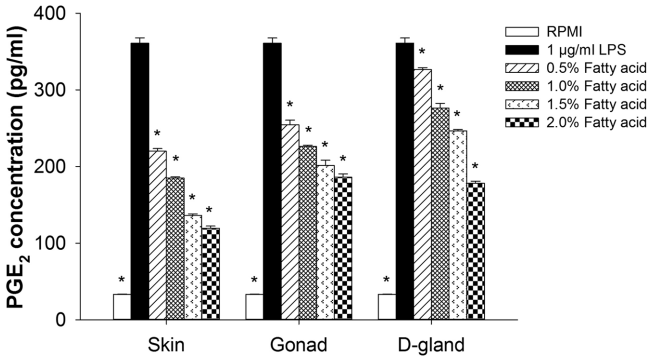

Fig. 4. Quantification of PGE_2 production from LPS-stimulated RAW264.7 cells cultured with different fatty acid concentrations (*$p < 0.01$).

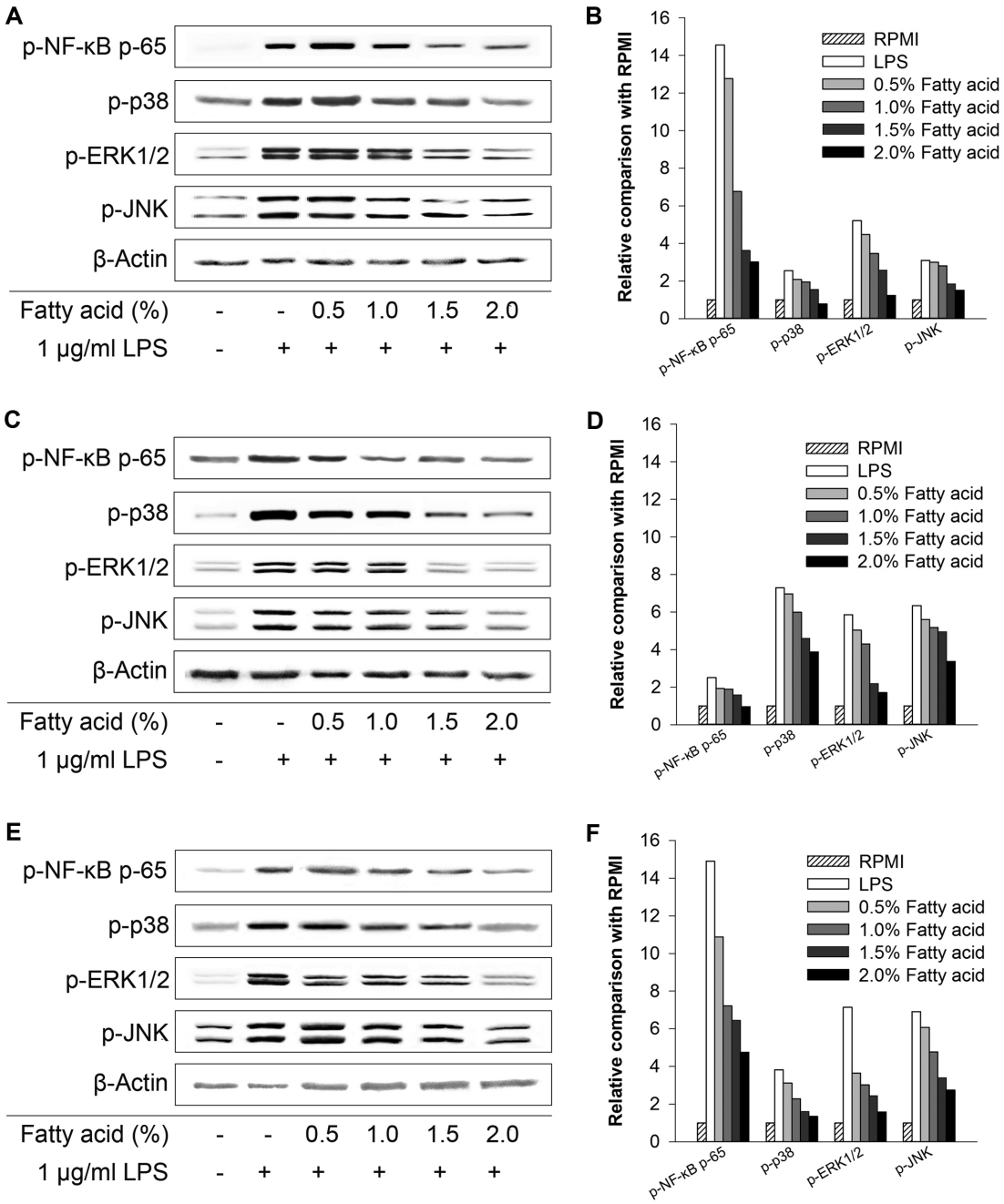

Fig. 5. Effect of *A. amurensis* fatty acids on proteins associated with NF-κB and MAPK pathways in LPS-stimulated RAW264.7 cells, (**A**) western blot of skin PUFAs, (**B**) relative band intensity of skin PUFAs, (**C**) western blot of gonad PUFAs, (**D**) relative band intensity of gonad PUFAs, (**E**) western blot of d-gland PUFAs, (**F**) relative band intensity of d-gland PUFAs.

and mitogen-activated protein kinase (MAPK) was examined. LPS-treated RAW264.7 cells were also used as a positive control and fatty acids from the three organs were used to treat the cells following stimulation with LPS. As shown Fig. 5, *A. amurensis* fatty acids dose-dependently inhibited the phosphorylation of NF-κB p-65 in the NF-κB signaling pathway. In contrast, *A. amurensis* fatty acids dose-dependently increased the level of IκBα (data not shown). Similar to the NF-κB signaling results, *A. amurensis* fatty acids inhibited the phosphorylation of extracellular signal-related kinase

(ERK1/2), c-Jun N-terminal kinase (JNK), and p38 in a dose–dependent manner. This result demonstrates that fatty acids from the three organs of *A. amurensis* inhibited inflammation through the MAPK and NF-κB signaling pathways in LPS-stimulated RAW264.7 cells.

Discussion

Asterias amurensis, a species of starfish widely distributed in the North Pacific Ocean as well as along the east coast of Korea, is having detrimental effects on the fishing industry [18]. Starfish including *A. amurensis* are regarded as a potential source of numerous bioactive materials including steroids, saponins, steroidal glycosides, anthraquinones, alkaloids, phospholipids, and peptides [19]. These compounds have diverse biological activities with anti-tumor, anti-inflammatory [20], immunomodulation, anti-allergy, anti-fungal, hemolytic [21], antimutagenic [22], neuritogenic [23], cytotoxic, and anti-viral [24] applications. However, no studies have examined the fatty acid profiles of *A. amurensis* internal organs and their immune-regulation effects on macrophage cells.

The current study revealed the fatty acid profiles of three organs; the skin, gonad, and d-gland, and their effects on immune molecules in RAW264.7 cells. The fatty acid profiles differed in different tissues. SFA appeared to be abundant in the skin rather than in the internal organs such as the gonad and d-gland; however, PUFA levels considerably differed by organ. The skin contained higher omega-6 PUFA levels than omega-3 PUFA levels, while the gonad and d-gland showed higher omega-3 PUFA than omega- 6 PUFA, suggesting that essential fatty acids levels differ depending on the tissue functions of marine organisms [35]. The d-gland contained the highest EPA levels among tissues, suggesting that the d-gland is an important organ that uses EPA and/or its metabolites. However, our current results revealed that d-gland fatty acid did not have a greater effect on anti-inflammatory regulation than other tissue fatty acids on LPS-stimulated RAW264.7 cells.

Macrophages are mainly involved in acute and chronic inflammatory responses, which produce the NO to enhance the ability of macrophages to kill antigens [36]. Therefore, fatty acids from the *A. amurensis* skin, gonad, and d-gland were evaluated to determine their cytotoxicity effects on macrophage RAW264.7 cells. The results showed that skin was not cytotoxic at any concentration, whereas high concentrations of gonad and d-gland fatty acids were cytotoxic. Additionally, NO, an important biomarker of inflammation in macrophage cells, and PGE_2, which plays a key role in inflammatory processes including pain, fever, swelling, and tenderness [37], were analyzed in macrophage cells. The results showed that NO was significantly decreased (Fig. 3) and PGE_2 was dramatically reduced (Fig. 4) when increasing concentrations of *A. amurensis* fatty acids were applied to LPS-stimulated RAW264.7 cells. Particularly, fatty acids from *A. amurensis* skin reduced NO and PGE_2 production more significantly than other tissues' fatty acids compared to LPS-stimulated cells as a positive control and unstimulated cells as a negative control. These results suggest that the skin of *A. amurensis* is a potential source of fatty acids among the three tissues tested.

In addition to NO and PGE_2 production, cytokines such as IL-1β, IL-6, and TNF-α cause pro-inflammatory reactions and are secreted by activated macrophages [38]. To regulate inflammation, NF-κB coordinates the expression of pro-inflammatory mediators, including iNOS, COX-2, and pro-inflammatory cytokines [39]. NF-κB is also activated or deactivated according to the phosphorylation and degradation of IκBα to regulate inflammatory reactions [40]. Moreover, MAPK-related molecules, including ERK1/2, JNK, and p38, play critical roles in regulating cell growth and differentiation and controlling cellular responses to cytokines and stresses [41]. MAPK pathways modulate NF-κB activation, and in turn promote the expression of pro-inflammatory cytokines and inflammatory processes [42, 43].

The current results demonstrated that fatty acids from *A. amurensis* tissues significantly decreased the expression of immune-associated genes such as *iNOS, IL-1β, IL-6,* and *TNF-α* as well as *COX-2* in LPS-stimulated RAW264.7 cells. The decreased expression levels led to further activation of NF-κB p-65 and MAPK molecules, such as ERK1/2, JNK, and p38, thus alleviating the immune response. These results therefore provide insight into the anti-inflammatory mechanism of *A. amurensis* fatty acids on immune cells and suggest that *A. amurensis* is a potential fatty acid source of anti-inflammation molecules.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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