

Anti-Inflammatory Effects of Stearidonic Acid Mediated by Suppression of NF- κ B and MAP-Kinase Pathways in Macrophages

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Abstract Stearidonic acid (SDA, 18:4n-3) is an omega-3 polyunsaturated fatty acid present in oils derived from plants of the Boraginaceae family. In this study, we determined the anti-inflammatory effects of SDA isolated from echium oil on lipopolysaccharide (LPS)-induced inflammatory responses in RAW 264.7 macrophages. SDA significantly downregulated the levels of the inducible nitric oxide synthase (iNOS) protein, thereby suppressing the production of nitric oxide (NO) in LPS-stimulated RAW 264.7 cells. In addition, SDA inhibited the nuclear translocation and promoter activity of nuclear factor κ B (NF κ B) and the phosphorylation of mitogen-activated protein kinases (MAPK) such as extracellular signal regulated kinase 1/2, c-jun N terminal kinase, and p38 in LPS-stimulated RAW 264.7 cells. Our results showed that SDA exerted anti-inflammatory effects by suppressing iNOS-mediated NO production via inactivation of NF κ B and MAPK signaling pathways.

Keywords Stearidonic acid · Echium oil · Anti-inflammatory · NF κ B (nuclear factor κ B) · MAPK (mitogen-activated protein kinases) pathway

Abbreviations

ALA	α -Linolenic acid
BSA	Bovine serum albumin
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified Eagle's medium
FBS	Foetal bovine serum
EPA	Eicosapentaenoic acid
ERK	Extracellular signal regulated kinase
JNK	c-jun N terminal kinase
iNOS	Inducible nitric oxide synthase
I κ B	Inhibitor of κ B
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MTT	3-(4,5-Dimethyl thiazol 2-yl)-2,5-diphenyltetrazolium
NO	Nitric oxide
NF κ B	Nuclear factor κ B
n-3 PUFA	Omega-3 polyunsaturated fatty acids
PBS	Dulbecco's phosphate-buffered saline
SDA	Stearidonic acid

Introduction

Dietary omega-3 polyunsaturated fatty acids (n-3 PUFA), primarily eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6), are beneficial in preventing chronic inflammatory diseases, including cardiovascular disease, coronary artery disease, and rheumatoid arthritis [1, 2]. Most epidemiological studies show a strong association between higher levels of plasma n-3 PUFA and lower in the levels of inflammatory mediators in the plasma [3–5]. It was reported that EPA and DHA reduced TNF- α and IL-1 β in LPS-activated human monocytes and murine

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macrophages [6, 7]. Fish oils are the major dietary source of EPA and DHA; however, overfishing or pollution of the marine environment has warranted the search for alternative plant-based n-3 PUFA [8, 9].

Stearidonic acid (SDA; 18:4, Fig. 1a) is a plant-based n-3 PUFA, which recently has received much attention because of its various physiological functions in the human body [10]. SDA has been reported to be more effective as a protecting agent in tumorigenesis of breast cancer cells than another plant-based PUFA, α -linolenic acid (18:3) [11]. Oils derived from the members of the Boraginaceae family, including *Echium* (viper's bugloss) are rich in SDA. *Echium* oil is an alternative natural source of n-3 PUFA, particularly SDA, which accounts for approximately 15–22% of total fatty acids [12]. However, the effect of SDA on inflammatory responses and its underlying molecular mechanism in LPS-stimulated RAW 264.7

macrophage cells have not been investigated. Therefore, this study aimed to investigate the anti-inflammatory effects of SDA isolated from echium oil.

Materials and methods

Materials

SDA (purity >99%) isolated from echium oil was provided by Dr. Inhwon Kim from Korea University (Seoul, Korea) [13]. DHA, fatty acid-free bovine serum albumin (BSA), LPS, 3-(4,5-dimethyl thiazol 2-yl)-2,5-diphenyltetrazolium (MTT), dimethyl sulfoxide (DMSO), and Griess reagent were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (PBS), foetal bovine serum (FBS), penicillin–streptomycin, and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). Antibodies for inducible nitric oxide synthase (iNOS), nuclear factor-kappa B (NF κ B), proliferating cell nuclear antigen (PCNA), and β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho or total antibodies to extracellular signal regulated kinase (ERK) 1/2, c-jun N terminal kinase (JNK), and p38 were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Peroxidase-conjugated secondary antibodies, NEPER[®] nuclear extraction kit and SuperSignal[®] West Pico chemiluminescent substrate were obtained from Thermo Scientific (Tewksbury, MA, USA).

Cell Culture and Treatment

RAW 264.7 cells (Koran Cell Line Bank, Seoul, Korea) were grown in DMEM supplemented with 10% FBS, 50 μ g/mL streptomycin, and 100 unit/mL penicillin and incubated at 37 °C in a humidified atmosphere of 5% CO₂. SDA (10 mM) or DHA (10 mM) as a positive control [7] were conjugated to 10% fatty acid-free BSA to ensure an even distribution in the cell culture media and were used for the treatment of cells. All stock solutions were filtered through a 0.2 μ m syringe filter and stored at –20 °C.

Cell Viability and Cytotoxicity

To determine cell viability, after treatment with SDA and DHA in the presence or absence of LPS (1 μ g/mL) for 18 h, cells were incubated in DMEM medium containing 0.5 mg/mL MTT for an additional 4 h at 37 °C; subsequently, DMSO was added to dissolve formazan. The absorbance was measured using a microplate reader (BioTek, Inc., Winooski, VT, USA). To measure cytotoxicity, the concentration of lactate dehydrogenase (LDH) was estimated

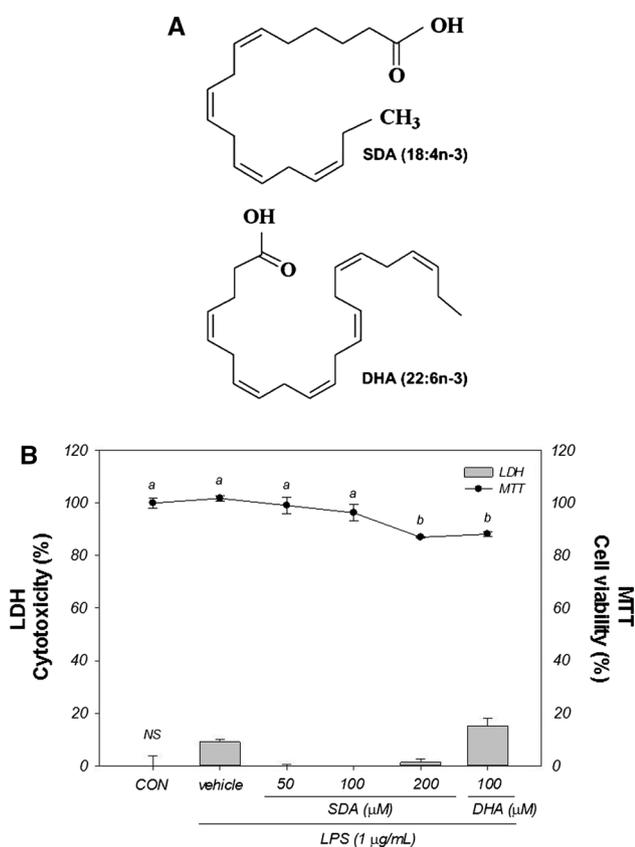


Fig. 1 Effect of stearidonic acid (SDA) on the cell viability and cytotoxicity in RAW 264.7 macrophages. **a** Chemical structure of SDA and docosahexaenoic acid (DHA). **b** RAW 264.7 cells were treated with different concentrations of SDA in the absence or presence of 1 μ g/mL lipopolysaccharide (LPS) for 18 h. DHA (100 μ M) was used as a positive control. Cell viability and cytotoxicity were determined using the MTT and LDH assays, respectively. Each value is presented as the mean \pm standard error ($n = 3$). NS not significant. Different letters among groups indicate significant differences obtained using Duncan's test ($P < 0.05$)

using an LDH cytotoxicity detection kit (Roche Applied Science, IN, USA).

Cellular Fatty Acid Analysis

Total lipids in RAW 264.7 cell lysates were extracted with chloroform/methanol (2:1, v/v). Fatty acid methyl esters by BF_3 -catalyzed transesterification were separated and quantified with a Hewlett-Packard 6890 gas chromatograph equipped with a fused-silica capillary column (SP-WAX, 60 m \times 0.25 mm i.d., Supelco, Bellefonte, PA, USA), auto injector, and flame-ionization detector (Hewlett-Packard, Anondale, PA, USA), as previously described [14].

Nitrite Determination

The levels of nitrite, a stable metabolite of NO, was measured using a colorimetric assay based on the Griess reaction, as previously described [15].

Western Blotting

To obtain whole cell extracts, the cells were washed with PBS and lysed in a Pro-Prep™ sample buffer (iNtRON Biotechnology, Seongnam, Korea). For nuclear extraction, cells were prepared using the NE-PER® nuclear extraction kit according to the manufacturer's guidelines. The protein concentration was quantified using the Take3™ Multivolume Plate (BioTek, Inc., Winooski, VT, USA) and equal amounts of proteins were electrophoretically transferred onto a nitrocellulose membrane following separation on a 10% sodium dodecylsulphate (SDS)-polyacrylamide gel. The membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBST) and subsequently incubated with various primary antibodies followed by addition of horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized on an X-ray film activated by chemiluminescence.

Luciferase Assay

RAW 264.7 macrophages were transferred using the Lipofectamine 2000 and pNF κ B-Luc reporter plasmid. Briefly, cells (1.5×10^5 cells/well) were seeded in a black 96-well plate. After the transfection, cells were treated with SDA or DHA in the presence or absence of LPS. We subsequently evaluated the luciferase activities by a Dual-Glo Luciferase assay kit (Promega, Madison, CA, USA) and a luminometer (Panomics, Fremont, CA, USA).

Statistical Analysis

The results are reported as mean \pm standard error (SE). Statistical comparisons were made using one-way analysis of variance (ANOVA) using SAS version 9.4 (SAS Institute, Cary, NC, USA), followed by Duncan's multiple comparison test. A p value <0.05 was considered statistically significant for all analyses.

Results and Discussion

Effects of SDA on Cell Viability and Cytotoxicity in LPS-Stimulated RAW 264.7 Cells

The MTT assay measures the mitochondrial activity of cells, which is an indicator of cell viability. The LDH leakage assay indicates membrane integrity, and this assay has been used as an indicator of cytotoxicity in various cell lines [16]. In this study, treatment with SDA did not affect the viability and did not induce cytotoxicity in RAW 264.7 macrophages (Fig. 1b). Although treatment with 200 μM SDA and 100 μM DHA in the presence of LPS induced a significant decrease in the cell viability of RAW 264.7 cells, the percentage of viable cells was more than 80%, and; thus, this treatment was non-cytotoxic [17]. Previous studies reported that the n-3 PUFA, such as DHA and SDA, were not cytotoxic at concentrations up to 200 μM in various cell culture systems [12, 18]. In addition, it was reported that 400 μM of SDA was non-cytotoxic to healthy dog peripheral blood mononuclear cells [19]. Similar dietary fatty acids such as EPA and DHA in humans could attain up to 500 μM in plasma after oral intake in diet [20, 21]. Thus, the effects of using SDA at 200 μM can be expected *in vivo*.

Effects of SDA on NO Production and iNOS Protein Expression in LPS-Stimulated RAW 264.7 Cells

Inflammation is a crucial body defense against pathogen invasion or endotoxin exposure. However, aberrant inflammatory responses contribute to the pathogenesis of various chronic inflammatory diseases [22]. iNOS-derived NO is one of the important mediators of macrophage-mediated inflammation [23]. We investigated the inhibitory effects of SDA on iNOS-derived NO production in LPS-stimulated RAW 264.7 macrophages and compared them with those obtained using DHA, a well-known anti-inflammatory n-3 PUFA. NO production and iNOS protein levels were markedly higher in cells treated with LPS than in the untreated control cells. However, treatment with SDA at 50–200 μM significantly

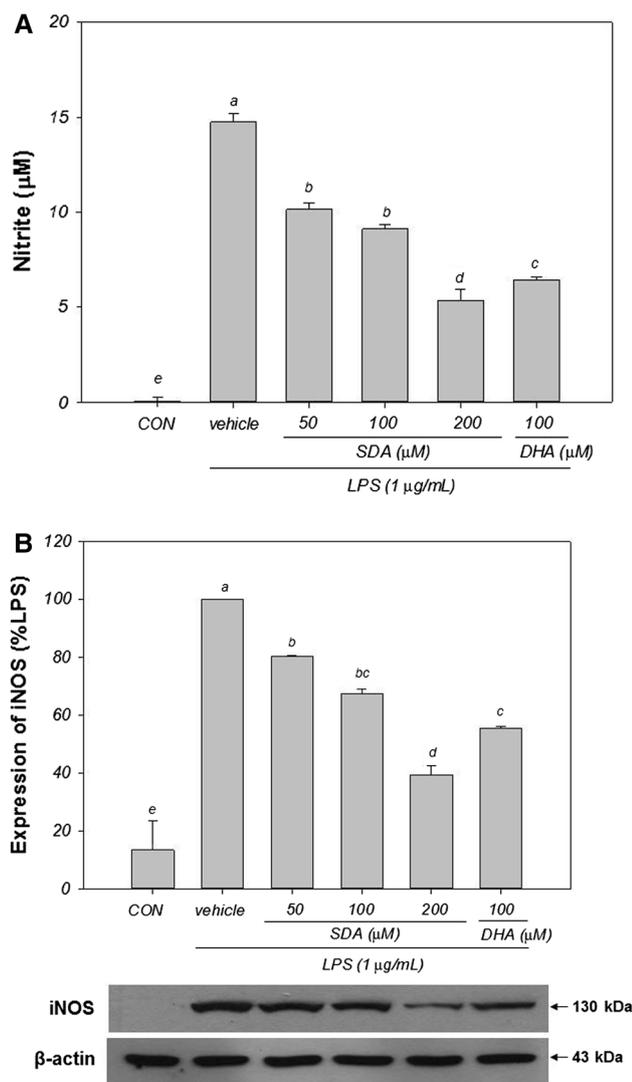


Fig. 2 Effect of stearidonic acid (SDA) on the production of inducible nitric oxide synthase (iNOS)-derived nitric oxide (NO) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. RAW 264.7 cells were treated with different concentrations of SDA in the absence or presence of 1 µg/mL LPS for 18 h. In addition, docosahexaenoic acid (DHA, 100 µM) was used as a positive control. **a** No production was determined by the level of nitrite formed in the medium using Griess reagent. **b** The expression of iNOS protein was measured using western blotting. The intensity of each band was quantified using Image J. The relative protein expression levels were normalised to those of β-actin, which was used as an internal control. Each value is presented as the mean ± standard error ($n = 3$). Different letters among groups indicate significant differences obtained using Duncan's test ($P < 0.05$)

reduced the LPS-induced NO production and iNOS protein levels (Fig. 2). Also, DHA at 100 µM significantly decreased LPS-induced NO production and iNOS protein levels. These results indicated that the inhibitory effects of SDA on LPS-induced NO production could be due to the downregulation of iNOS expression.

Effects of SDA on Nuclear Translocation and Transcription Activity of NFκB in LPS-Stimulated RAW 264.7 cells

NFκB is a key transcription factor that regulates the expression of genes involved in the immune and inflammatory responses. iNOS expression is closely related to the upregulation of NFκB in LPS-stimulated macrophages [24]. NFκB normally exists in the cytoplasm as the p50/p65 heterodimers complexed with the inhibitor of κB (IκB) proteins. Upon activation by inflammatory stimuli such as LPS, NFκB subunits are released from IκBα and translocate to the nucleus, where they bind to the promoters of inflammation-related genes, which ultimately leads to the transcription of target genes, including iNOS [25]. Treatment with LPS increased the nuclear protein expression of p65 and p50 NFκB subunits and the promoter activity, while SDA markedly inhibited the LPS-induced NFκB translocation and the promoter activation (Fig. 3). SDA and DHA have similar effects on the protein expression levels of NFκB at the same concentration of 100 µM. Furthermore, DHA also significantly decreased the nuclear translocation of NFκB and the promoter activity in LPS-stimulated macrophages. These results suggested that the suppression of the NFκB pathway by SDA might be a pivotal mechanism governing the regulation of pro-inflammatory mediators.

Effects of SDA on MAPK Kinases in LPS-Stimulated RAW 264.7 Cells

The mitogen-activated protein kinases (MAPK), including ERK1/2, JNK, and p38 subfamilies, are one of the intracellular signaling cascades that play a critical role in the inflammatory process [26]. Activation of MAPK influences iNOS-derived NO production via control of the activation of NFκB in LPS-stimulated macrophages [27]. The phosphorylation levels of ERK1/2, JNK, and p38 increased after exposure to LPS, but SDA treatments effectively decreased this LPS-induced phosphorylation in the cells (Fig. 4). Further, DHA significantly decreased the phosphorylation of ERK1/2, JNK, and p38 in LPS-stimulated macrophages. These results indicated that inhibition of activation of all three MAPK may be the mechanism underlying the anti-inflammatory effect of SDA involved.

Effects of SDA on Cellular Fatty Acid Profile in LPS-Stimulated RAW 264.7 Cells

Treatment of LPS-stimulated RAW 264.7 macrophages with 100 µM SDA demonstrated a significant increase in the concentrations of SDA (18:4n-3) and EPA (20:5n-3) in the cellular lipids (Table 1). Treatment with SDA did not result in significantly increased appearance of DHA

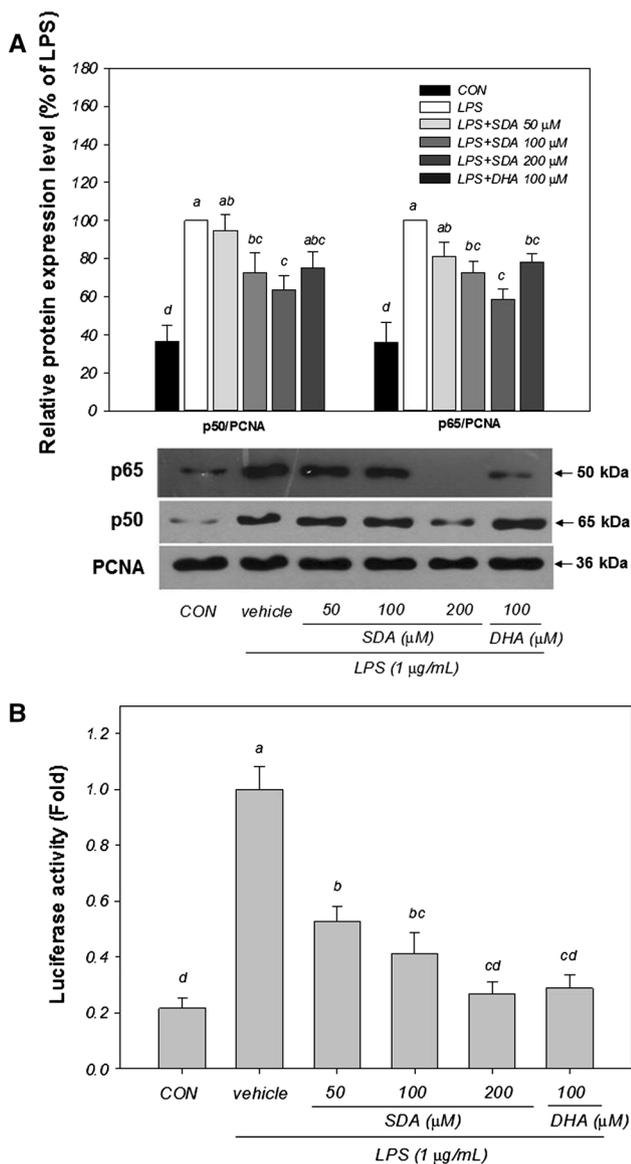


Fig. 3 Effect of stearidonic acid (SDA) on the nuclear translocation and promoter activity of nuclear factor kappa B (NFκB) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. **a** RAW 264.7 cells were treated with different concentrations of SDA in the absence or presence of 1 μ g/mL LPS for 1 h 30 min. Docosahexaenoic acid (DHA, 100 μ M) was used as a positive control. Nuclear extracts were isolated, and the protein levels of the NFκB subunit were determined using western blotting. The intensity of each band was quantified using Image J. The relative protein expression levels were normalised to those of PCNA, which was used as an internal control. **b** NFκB promoter activity was determined by luciferase reporter assay. Each value is presented as the mean \pm standard error ($n = 3$). Different letters among groups indicate significant differences obtained using Duncan’s test ($P < 0.05$)

(22:6n-3) in LPS-induced RAW 264.7 cells, but treatment with 100 μ M DHA had very high levels of DHA. In the metabolic pathway for n-3 PUFA synthesis, SDA is the metabolite of the rate-limiting Δ -6-desaturase (D6D) step

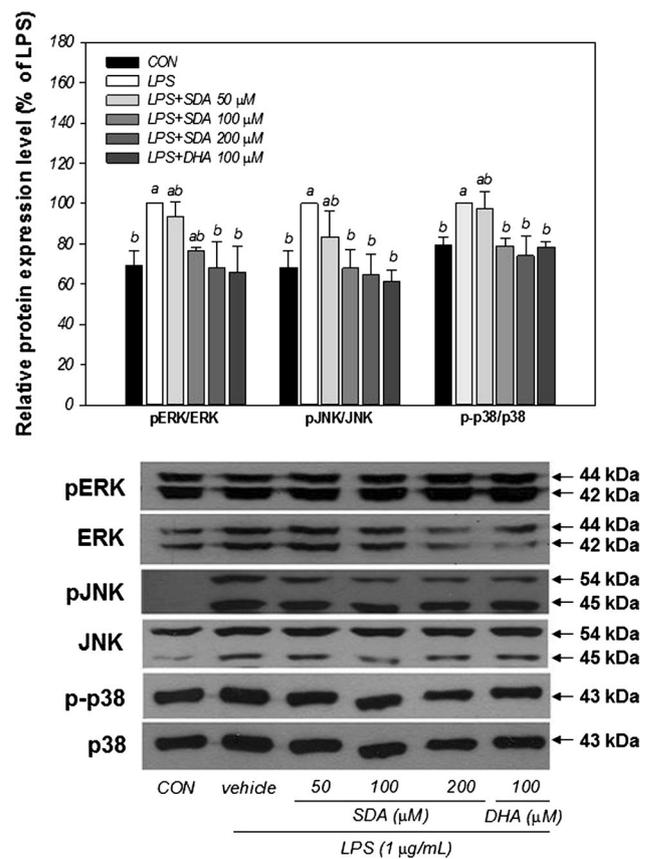


Fig. 4 Effect of stearidonic acid (SDA) on the phosphorylation of mitogen-activated protein kinases (MAPK) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. RAW 264.7 cells were treated with different concentrations of SDA in the absence or presence of 1 μ g/mL LPS for 30 min. Docosahexaenoic acid (DHA, 100 μ M) was used as a positive control. The protein levels of MAPK were determined using western blotting. The intensity of each band was quantified using Image J. The relative protein expression levels were normalised to those of total MAPK. Each value is presented as the mean \pm standard error ($n = 3$). Different letters among groups indicate significant differences obtained using Duncan’s test ($P < 0.05$)

Table 1 Fatty acid composition of RAW 264.7 macrophages cells treated with stearidonic acid (SDA) or docosahexaenoic acid (DHA)

Fatty acids	Treatment group			
	CON	LPS	LPS + SDA	LPS + DHA
18:4n-3	–	–	6.263	–
20:5n-3	0.903 ^{b,1}	0.184 ^b	2.776 ^a	0.343 ^b
22:6n-3	4.421 ^b	1.612 ^b	1.341 ^b	23.230 ^a

Cells were coincubated for 18 h with 100 μ M SDA or 100 μ M DHA with 1 μ g/mL lipopolysaccharide

All values are means of triplicate analysis

Concentration of fatty acids was expressed as g/100 g

¹ Mean values in a row followed by different superscripts letters are significantly different by Duncan’s multiple range test ($P < 0.05$)

in the biosynthesis from α -linolenic acid (ALA; 18:3) to EPA [27]. Several studies have suggested that SDA could be more effectively converted to EPA than ALA in the body because it does not require the initial step catalyzed by the D6D step [28]. In this study, our data indicated that SDA might be readily converted to EPA, but not DHA. However, this conversion may not achieve EPA levels adequate for anti-inflammatory effects.

Previous observational studies strongly support the efficacy of the marine-based n-3 PUFA such as DHA for preventing inflammation [29]. It has been reported particularly that DHA mediate its anti-inflammatory via the NF κ B signaling system in a macrophage study [7]. In this study, SDA, a plant-based n-3 PUFA, is as effective as DHA against inflammatory response in LPS-stimulated macrophages. Thus, our results suggest that SDA isolated from echium oil may be as an alternative to the fish-based fatty acids for preventing inflammatory diseases.

Conclusion

In conclusion, our results indicate that SDA alleviates LPS-induced inflammatory events by inhibiting the activation of NF κ B and MAPK. This study provides a molecular basis for understanding the inhibitory effects of SDA on inflammation.

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