Docosapentaenoic Acid (22:5n-3) Downregulates mRNA Expression of Pro-inflammatory Factors in LPS-activated Murine Macrophage Like RAW264.7 Cells

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Abstract: Docosapentaenoic acid (22:5n-3, n-3 DPA) is a n-3 polyunsaturated fatty acid (PUFA) found in fish oil, and has been reported to have health benefits. This study investigated conversion of n-3 DPA, and examined the anti-inflammatory effects of n-3 DPA on activated macrophages. Murine macrophage-like RAW264.7 cells were incubated in culture media containing n-3 DPA for 72 h. The level of n-3 DPA in the fatty acid composition of the total lipid fraction increased in a dose-dependent manner. Furthermore, the levels of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were higher in treated cells than in control cells. In RAW264.7 cells stimulated by lipopolysaccharide (LPS), n-3 DPA significantly down-regulated mRNA expression of pro-inflammatory factors such as IL-6, IL-1β, iNOS and COX-2. Production of IL-6 was also reduced by n-3 DPA in a dose-dependent manner. We found that n-3 DPA treatment resulted in greater IL-6 mRNA down-regulation than that achieved with EPA treatment, and was similar to that of DHA treatment. Furthermore, expression levels of IL-6 and IL-1β mRNAs were measured in the presence of the delta-6 desaturase inhibitor SC26196 in the culture medium to inhibit the conversion of n-3 DPA to DHA. There was no significant difference in the down-regulation in the mRNA expression of pro-inflammatory cytokines in RAW264.7 cells by n-3 DPA with or without presence of SC26196. These results demonstrate that n-3 DPA exhibits anti-inflammatory effects in activated RAW264.7 cells, which are independent of DHA conversion.

Key words: n-3 docosapentaenoic acid, anti-inflammation, macrophage, n-3 polyunsaturated fatty acids, cytokine

1 INTRODUCTION

Long chain n-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are well known for their beneficial health effects. These n-3 PUFAs are major fatty acids in fish oil. In particular, DHA is enriched in tuna fish, and EPA in sardines. Docosapentaenoic acid (22:5n-3, n-3 DPA) is the third most prevalent n-3 PUFA in marine oil. Seal meat, salmon and abalone contain n-3 DPA at comparatively high levels, approximately 2-7% of total fatty acid composition¹,². Furthermore, n-3 DPA levels in human milk have been reported to be higher than EPA levels, and are comparable to DHA levels³.

In previous studies, n-3 DPA has been reported to inhibit angiogenesis⁴ and platelet aggregation⁵, and to improve lipid metabolism in both in vitro and in vivo models⁶⁻⁸. In epidemiological investigations of older adults, higher n-3 DPA levels in the blood have been correlated with lower total mortality, including death from coronary heart disease⁹. However, in comparison to the vast number of studies on EPA and DHA, information regarding the health benefits of n-3 DPA is very limited.

Previous human and rodent studies suggest that dietary n-3 DPA increases not only the concentration of n-3 DPA in plasma triglycerides, but also the levels of both EPA and DHA¹⁰,¹¹. Recently, we reported that the bioconversion profile of n-3 DPA is different among human cell lines established from different tissues and blood. We found that n-3 DPA was converted to DHA in HepG2 cells (hepatoblastoma cell line), but not in Caco-2 (colon carcinoma cell line) or THP-1 (monocytic leukemia cell line) cells, even though retro-conversion of n-3 DPA to EPA was observed.
in all three cell lines\(^{12}\). This metabolic information is important when considering the nutritional roles of n-3 DPA.

Chronic low-grade inflammation has been identified as the primary trigger for the pathogenesis of metabolic syndrome, cardiovascular diseases and cancers\(^{13-15}\). During the development of inflammation, production of pro-inflammatory cytokines and chemokines such as interleukin (IL)-1\(\beta\), IL-6, monocyte chemoattractant protein-1 (MCP-1) and regulated on the activation of normal T-cell expressed and secreted (RANTES) increases due to immune cell activation\(^{16,17}\). Macrophages play a crucial role in the regulation and production of cytokines and chemokines\(^{18}\). Furthermore, cyclooxygenase-2 (COX-2) and nitric oxide synthase (NOS) produce inflammatory mediators such as prostaglandin E\(_2\) and nitric oxide (NO), respectively\(^{19,20}\). Therefore, suppression of pro-inflammatory factors production in activated macrophages is very important for the prevention of inflammatory-related diseases.

Herein, we report the anti-inflammatory effect of n-3 DPA on murine macrophage-like RAW264.7 cells activated by lipopolysaccharide (LPS). Initially, the conversion of n-3 DPA in RAW264.7 cells was evaluated. The anti-inflammatory effect of n-3 DPA was then investigated and compared with those of EPA and DHA. In RAW264.7 cells, n-3 DPA can be converted to both DHA and EPA. We utilized the inhibitor SC26196 to stop the conversion of n-3 DPA to DHA and evaluated the effect of n-3 DPA on IL-6 and IL-1\(\beta\) mRNA expression in the presence of this delta-6 desaturase inhibitor. The present results showed that down-regulation of IL-6 mRNA expression by n-3 DPA was more potent than that by EPA, and similar as that by DHA. Furthermore, n-3 DPA exhibited anti-inflammatory effects in LPS-activated RAW264.7 cells independent of its conversion to DHA.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

The fatty acid n-3 DPA, EPA and DHA were purchased from Cayman Chemical (Ann Arbor, MI, USA). Fetal bovine serum (FBS), RPMI 1640 medium, penicillin and streptomycin were obtained from Gibco\(^{8}\), Thermo Fisher Scientific Inc. (Waltham, MA, USA). LPS and the delta-6 desaturase inhibitor (SC26196(\(\alpha,\alpha\)-diphenyl-4-[(3-pyridinylmethylene)amino]-1-piperezinopentanenitrile)) were purchased from Sigma Chemical (St. Louis, MO, USA). \(\alpha\)-Tocopherol was purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2 Cell culture

Murine macrophage-like cell line RAW264.7 was purchased from DS Pharma Biomedical (Suita, Osaka, Japan). RAW264.7 cells \((2 \times 10^5\) cells/well\) were pre-incubated in 12-well plates with 1 mL RPMI 1640 containing 10% FBS, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin. Cells were maintained at 37\(^\circ\)C in humidified atmosphere containing 5% CO\(_2\) for 48 h. n-3 DPA, EPA, or DHA (25-75 \(\mu\)M) were added into the culture medium, and the cells were incubated for 72 h. The delta-6 desaturase inhibitor, SC26196 (1 \(\mu\)M) was added into the cultured medium 3 h prior to the addition of n-3 DPA (at 45 h pre-incubation). n-3 PUFAs and SC26196 were added as ethanol and dimethyl-sulfoxide (DMSO) solutions, respectively. Final concentrations of ethanol and DMSO were adjusted to 0.1% in the culture medium. Then, inflammation in cells was induced by LPS (0.1 \(\mu\)g/mL of final concentration) for 6 h in the presence of n-3 PUFAs and/or SC26196. To prevent n-3 PUFAs oxidation, 1 \(\mu\)M \(\alpha\)-tocopherol was added in the culture medium during n-3 PUFAs treatment.

2.3 Extraction of cellular lipids and fatty acid analysis

RAW264.7 cells \((1 \times 10^5\) cells/dish\) were pre-incubated in a 10 mm dish with 10 mL RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin at 37\(^\circ\)C in humidified atmosphere containing 5% CO\(_2\) for 48 h. Treatments with n-3 PUFAs and/or SC26196 were conducted as described in section 2.2 Cell culture. Following incubation for 72 h with n-3 PUFAs and/or SC26196, RAW264.7 cells were washed three times with phosphate-buffered saline (PBS), and were centrifuged at 115 \(\times\) g for recovery. Total lipid was extracted from the cells by the Bligh and Dyer method\(^{21}\). Fatty acid compositions of cellular lipids were determined by gas chromatography (GC); methyl ester derivatives were prepared according to procedures described in a previous paper\(^{22}\). GC analysis was performed using a Shimadzu GC-2014 gas chromatograph equipped with a flame-ionization detector (FID) and Omegawax-320 capillary column \((30\) m \(\times\) 0.32 mm i.d.: Supelco, Bellefonte, PA, USA). The temperatures of the detector, the injector, and the column were 260, 250 and 200\(^\circ\)C, respectively. The carrier gas, helium, flow was at 50 kPa. The split ratio was 1:49 (v/v). Each fatty acid was identified based on the retention times of authentic standards.

2.4 Quantitative real-time RT-PCR

RAW264.7 cells \((2 \times 10^5\) cells/well\) were seeded into 12-well plates with 1 mL RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin. Cells were incubated at 37\(^\circ\)C in humidified atmosphere containing 5% CO\(_2\) for 48 h. Fatty acids (n-3 DPA, EPA or DHA at 25-75 \(\mu\)M) were added into the culture medium together with 1 \(\mu\)M \(\alpha\)-tocopherol, and cells were further incubated for 72 h. Total RNA was extracted from the cells with the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer’s protocol. Then, cDNA was synthesized from total RNA using the High-Ca-
pacity cDNA Archive Kit (Applied Biosystems Japan Ltd, Tokyo, Japan). Quantitative real time RT-PCR was performed on the ABI Prism 7500 (Applied Biosystems Japan Ltd, Tokyo, Japan). Cycling conditions for PCR were as follows; 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. PCR primers and TaqMan® probes were obtained from TaqMan® Gene Expression Assays (Applied Biosystems Japan Ltd, Tokyo, Japan); IL-6: Mm00446190_m1, IL-1β: Mm00434228_m1, COX-2: Mm00478374_m1, iNOS: Mm00440502_m1; MCP-1: Mm00441242_m1, RANTES: Mm01302427_m1, GAPDH: Mm99999915_g1.

2.5 Enzyme-linked immunosorbent assay

The concentration of IL-6 in the culture medium of RAW264.7 cells was measured by enzyme-linked immunosorbent assay (ELISA) using commercialized kits (Thermo Scientific, Frederick, MD, USA) according to the manufacturer’s protocol.

2.6 Statistical Analysis

All values are expressed as mean ± SE (n = 3). Statistical differences were determined by the Scheffe’s test at p < 0.05.

### 3 RESULTS

3.1 Fatty acid composition of total lipid in RAW264.7 cells treated with n-3 DPA

RAW264.7 cells were incubated with 25-75 μM n-3 DPA for 72 h, and fatty acid composition was determined by GC-FID. The percentage of n-3 DPA in the cells increased in a dose-dependent manner, and reached 13.34 ± 0.65% with 75 μM n-3 DPA (Table 1). Cytotoxicity was not observed by the treatment with 75 μM n-3 DPA for 72 h (data not shown). Conversely, n-3 DPA in control cells (treated with ethanol) was found to be 1.99 ± 0.09%. The percentage of EPA and DHA also significantly increased to 3.53 ± 0.35% and 10.23 ± 0.27%, respectively, with 75 μM n-3 DPA, although 20:4n-6 level was reduced. In addition, 16:0 and 18:0 increased, and 16:1, 18:1n-9 and 18:1n-7 decreased in the cells treated with n-3 DPA (Table 1).

3.2 Effect of n-3 PUFAs on the expression of pro-inflammatory cytokines in RAW264.7 cells

The effects of n-3 DPA on mRNA expression of pro-inflammatory factors and protein production in RAW264.7 cells were investigated. Expression of IL-6, IL-1β, MCP-1, and RANTES mRNAs induced by LPS stimulation was significantly suppressed by n-3 DPA (Fig. 1). Furthermore, n-3 DPA treatment in RAW264.7 cells markedly decreased mRNA expressions of iNOS and COX-2, which produce the inflammatory factors NO and PGE2. Reduction of IL-6 con-

### Table 1  Fatty acid composition of total lipid extracted from RAW264.7 cells treated with n-3 DPA.

| (%)          | Control       | n-3 DPA 25 μM | n-3 DPA 50 μM | n-3 DPA 75 μM |
|--------------|---------------|---------------|---------------|---------------|
| 14:0         | 1.06 ± 0.04a  | 1.26 ± 0.07a  | 1.36 ± 0.06b  | 1.46 ± 0.02b  |
| 16:0         | 4.56 ± 0.06c  | 3.65 ± 0.03b  | 21.46 ± 0.48c | 21.93 ± 0.68c |
| 18:0         | 16.89 ± 0.38c | 19.44 ± 0.12b | 19.36 ± 0.45b | 18.46 ± 0.07b |
| 18:1n-9      | 21.92 ± 0.53c | 15.02 ± 0.45b | 10.33 ± 0.31c | 9.13 ± 0.25c  |
| 18:1n-7      | 10.21 ± 0.14c | 8.40 ± 0.14b  | 6.83 ± 0.18c  | 6.12 ± 0.03c  |
| 20:3n-6      | 1.01 ± 0.01c  | 1.15 ± 0.03b  | 1.16 ± 0.02b  | 1.15 ± 0.03b  |
| 20:4n-6      | 2.22 ± 0.03c  | 0.39 ± 0.03b  | 0.22 ± 0.02c  | 0.15 ± 0.01c  |
| 20:2n-6      | 4.53 ± 0.06c  | 1.81 ± 0.17b  | 0.82 ± 0.04c  | 0.51 ± 0.07c  |
| 20:3n-3      | 0.56 ± 0.03c  | 0.45 ± 0.08b  | 0.52 ± 0.05a  | 0.58 ± 0.01a  |
| 20:4n-3      | 6.22 ± 0.11c  | 5.56 ± 0.14b  | 5.23 ± 0.09b  | 5.09 ± 0.06b  |
| 20:5n-3 (EPA)| 0.16 ± 0.00c  | 0.93 ± 0.14b  | 2.20 ± 0.16c  | 3.53 ± 0.35c  |
| 22:5n-3 (n-3 DPA) | 1.99 ± 0.09c | 10.68 ± 0.28c | 12.16 ± 0.24c | 13.34 ± 0.65c |
| 22:6n-3 (DHA)| 2.43 ± 0.14c  | 6.09 ± 0.19c  | 8.81 ± 0.25c  | 10.23 ± 0.27d |

RAW264.7 cells were treated with 25-75 μM n-3 DPA for 72 h. Control cells were treated with ethanol. Data are presented as means ± SE, n = 3. Different letters denote significant differences at p < 0.05 (Scheffe’s F test).
The reduction was dose-dependent.

In the present study, we further compared mRNA expression and protein production of IL-6 in RAW264.7 cells treated with EPA, n-3 DPA and DHA. All of the n-3 PUFAs...
markedly suppressed mRNA expression of IL-6 as compared to LPS+ cells without n-3 PUFA treatment (Fig. 3). Treatment with n-3 DPA and DHA resulted in significantly greater down-regulated IL-6 mRNA expression as compared to EPA treatment. In addition, IL-6 concentration in the culture medium was significantly reduced by EPA, n-3 DPA, and DHA to 1.92 + 0.09, 1.18 + 0.23 and 0.88 + 0.24 ng/mL, respectively, as compared with 6.52 + 0.52 ng/mL observed in control RAW264.7 cells (Table 2). The suppressive effect of DHA on IL-6 secretion was greater than that of EPA, and was similar to that of n-3 DPA.

3.3 Anti-inflammatory effects of n-3 DPA under delta-6 desaturase inhibition

Our results showed that n-3 DPA was incorporated into RAW264.7 cells, and was partly converted to DHA and EPA (Table 1). Therefore, it is unclear whether the suppressive effect of n-3 DPA on LPS-induced mRNA expression of pro-inflammatory cytokines is dependent on n-3 DPA or the converted DHA. To examine the anti-inflammatory effect of n-3 DPA in detail, we measured mRNA expression levels of pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells treated with a delta-6 desaturase inhibitor.

Addition of the delta-6 desaturase inhibitor SC26196 in the culture medium completely inhibited the bioconversion of n-3 DPA to DHA, and led to the accumulation of n-3 DPA in RAW264.7 cells (Fig. 4A). Treatment with n-3 DPA suppressed LPS-induced mRNA expression of IL-6 and IL-1β even in the presence of SC26196 (Fig. 4B and 4C). In addition, no significant difference was observed in n-3 DPA-mediated reduction of IL-6 and IL-1β mRNA expressions following LPS-stimulation in RAW264.7 cells treated with or without SC26196. These findings indicate that the anti-inflammatory effect of n-3 DPA is, at least partially, inde-
DISCUSSION

The fatty acid, n-3 DPA, is one of the intermediate fatty acids produced during the conversion of EPA to DHA via elongation, desaturation, and \( \beta \)-oxidation of 24:6(n-3). In a previous study, we have reported that the metabolism of n-3 DPA is different in human cell lines. In HepG2 cells, n-3 DPA was converted to DHA. However, it was not converted in Caco-2 or THP-1 cells, although retro-conversion to EPA was observed in all three cell lines\(^{12}\). In the present study, we analyzed the conversion of n-3 DPA in murine macrophage-like RAW264.7 cells. RAW264.7 cells were incubated in culture media containing 25-75 \( \mu \)M n-3 DPA for 72 h, and n-3 DPA level increased in a dose-dependent manner. Furthermore, both DHA and EPA levels also increased when compared to those of control cells.

Chronic inflammation is well known to be involved in the onset of metabolic syndrome and cardiovascular disease\(^{13,14}\). Macrophages play a crucial role in inflammation and immune functions, including cytokine production and induction of pro-inflammatory-associated enzymes\(^{18-20}\). The n-3 fatty acids, EPA and DHA, have been reported to exhibit anti-inflammatory effects through suppression of inflammatory factors produced in macrophages\(^{23,24}\). As compared with DHA and EPA, there is little known regarding the anti-inflammatory effects of n-3 DPA on macrophages. Therefore, in the present study, the regulation of
mRNA expression and protein secretion of pro-inflammatory factors by n-3 DPA was investigated using LPS-stimulated RAW264.7 cells. In addition, we compared the anti-inflammatory effects of n-3 DPA with DHA and EPA.

We demonstrated that n-3 DPA reduced mRNA expression of pro-inflammatory factors such as IL-6, IL-1β, MCP-1, RANTES, INOS and COX-2 in LPS-stimulated RAW264.7 cells. Furthermore, n-3 DPA treatment suppressed LPS-induced IL-6 production in RAW264.7 cells in a dose-dependent manner. These results indicate that n-3 DPA is a highly potent fatty acid for attenuating the production of inflammatory factors through down-regulation of mRNA expression.

We further compared the anti-inflammatory effects of n-3 DPA, EPA, and DHA on LPS-activated RAW264.7 cells. All three fatty acids significantly suppressed IL-6 mRNA expression and protein production in LPS-stimulated RAW264.7 cells. It is noteworthy that down-regulation of IL-6 mRNA expression in cells treated with n-3 DPA and DHA was significantly greater than that in EPA-treated cells. Furthermore, IL-6 concentration in the culture medium of cells incubated with DHA was significantly lower than that of EPA and was similar to n-3 DPA. These results suggest that DHA and n-3 DPA are more effective than EPA in alleviating LPS-induced pro-inflammatory factor production and mRNA expression in RAW264.7 cells. Weldon et al. reported that DHA exhibits greater anti-inflammatory effect on human THP-1 macrophages as compared to that of EPA. Norris and Dennis reported that EPA indirectly inhibits COX through elongation to n-3 DPA. However, no information regarding the ability of n-3 DPA to regulate mRNA expression of pro-inflammatory factors in activated macrophages was described. The present results demonstrate that n-3 DPA is a highly potent anti-inflammatory fatty acid in LPS-stimulated RAW264.7 cells.

In RAW264.7 cells treated with n-3 DPA, levels of DHA and EPA, as well as n-3 DPA, were significantly increased. However, it was unclear whether down-regulation of IL-6 mRNA expression by n-3 DPA in LPS-induced RAW264.7 cells was dependent on the conversion of n-3 DPA to DHA or due to the n-3 DPA itself. Therefore, we examined the anti-inflammatory effect of n-3 DPA in the presence of the delta-6 desaturase inhibitor SC26196, which inhibits the n-3 DPA to DHA conversion. n-3 DPA significantly down-regulated mRNA expressions of IL-6 and IL-1β in the presence of SC26196, to the same degree as that observed by n-3 DPA treatment alone. These results indicate that n-3 DPA can directly exert health benefits via its anti-inflammatory properties that are independent of conversion to DHA.

In previous studies, it has been reported that DHA regulates the production of pro-inflammatory mediators through inhibition of NF-κB activation. In addition, n-3 DPA-derived 7, 8, 17-trihydroxy-9, 11, 13, 15E, 19Z-docosapentaenoic acid (RvD1(17,19)) and 7, 14-dihydroxy-8, 10, 12, 16Z, 19Z-docosapentaenoic acid (MaR1(17,19)) have been identified as specialized mediators that act to resolve inflammation. Further research is required to clarify the molecular mechanism underlying the anti-inflammatory effects of n-3 DPA.

5 CONCLUSION

Treatment of RAW264.7 cells with n-3 DPA led to an accumulation of n-3 DPA, as well as an increase in DHA and EPA. n-3 DPA significantly down-regulated mRNA expression of pro-inflammatory factors and reduced IL-6 production. The down-regulation of IL-6 mRNA by n-3 DPA was similar as that induced by DHA, and more effective than EPA. As the down-regulation of mRNA expression of pro-inflammatory cytokines by n-3 DPA was unaffected by the addition of delta-6 desaturase inhibitor SC26196, we conclude that the anti-inflammatory effect of n-3 DPA is dependent on its original form, rather than on its converted DHA in the cells.

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