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Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid

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Abstract

A number of studies have investigated the effects of fish oil on the production of pro-inflammatory cytokines using peripheral blood mononuclear cell models. The majority of these studies have employed heterogeneous blends of long-chain $n-3$ polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which preclude examination of the individual effects of LC $n-3$ PUFA. This study investigated the differential effects of pure EPA and DHA on cytokine expression and nuclear factor κB (NF-κB) activation in human THP-1 monocyte-derived macrophages. Pretreatment with 100 μM EPA and DHA significantly decreased lipopolysaccharide (LPS)-stimulated THP-1 macrophage tumor necrosis factor (TNF) α, interleukin (IL) 1β and IL-6 production ($P<.02$), compared to control cells. Both EPA and DHA reduced TNF-α, IL-1β and IL-6 mRNA expression. In all cases, the effect of DHA was significantly more potent than that of EPA ($P<.01$). Furthermore, a low dose (25 μM) of DHA had a greater inhibitory effect than that of EPA on macrophage IL-1β ($P<.01$ and $P<.04$, respectively) and IL-6 ($P<.003$ and $P<.003$, respectively) production following 0.01 and 0.1 μg/ml LPS stimulation. Both EPA and DHA down-regulated LPS-induced NF-κB/DNA binding in THP-1 macrophages by ~13% ($P\leq.03$). DHA significantly decreased macrophage nuclear p65 expression ($P\leq.05$) and increased cytoplasmic IκBα expression ($P\leq.05$). Although similar trends were observed with EPA, they were not significant. Our findings suggest that DHA may be more effective than EPA in alleviating LPS-induced pro-inflammatory cytokine production in macrophages — an effect that may be partly mediated by NF-κB. Further work is required to elucidate additional divergent mechanisms to account for apparent differences between EPA and DHA.

Keywords: Macrophage; Eicosapentaenoic acid; Docosahexaenoic acid; Cytokines; Nuclear factor κB

1. Introduction

Fish oil is rich in long-chain $n-3$ polyunsaturated fatty acids (LC $n-3$ PUFA) eicosapentaenoic acid (EPA; 20:5$n-3$) and docosahexaenoic acid (DHA; 22:6$n-3$). Research indicates that LC $n-3$ PUFA supplementation has a beneficial effect on inflammatory symptoms in diseases such as atherosclerosis, rheumatoid arthritis and inflammatory bowel disease [1–3]. However, the mechanisms by which LC $n-3$ PUFA exert beneficial effects in disease states remain unclear. Fish oil supplementation has been reported to decrease ex vivo production of the classical pro-inflammatory cytokines tumor necrosis factor (TNF) α, interleukin (IL) 1β and IL-6 in human and animal models [4–8].

The majority of studies investigating the immunomodulatory effects of LC $n-3$ PUFA to date have employed fish oils, which contain a heterogeneous blend of EPA and DHA. The use of such mixtures of LC $n-3$ PUFA in fish oil capsules, emulsions and animal feeds has limited our understanding of the individual effects of EPA and DHA and has prevented a direct comparison of the relative potencies of these fatty acids. Only a limited number of

Abbreviations: DON, deoxynivalenol; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC PUFA, long-chain polyunsaturated fatty acids; NF-κB, nuclear factor κB; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride.

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studies have examined the effects of feeding pure EPA and/or DHA in vivo, and the findings reported to date are conflicting [9]. Many human supplementation studies have examined the effects of fish oil consumption on ex vivo peripheral blood mononuclear cell cytokine production. Consequently, studies of LC $n-3$ PUFA and cytokine production in pure human macrophage populations are limited. The critical role of monocytes and monocyte-derived macrophages as mediators of innate and adaptive immunity makes these cells an important but neglected target for the anti-inflammatory effects of LC $n-3$ PUFA in humans.

Therefore, the objective of this study was to compare the individual effects of EPA and DHA on human macrophage-derived mediators of inflammation in the THP-1 macrophage cell model. The effects of EPA and DHA on TNF-α, IL-1β and IL-6 mRNA expression and production were investigated in THP-1 monocyte-derived macrophages. There is evidence to suggest that LC $n-3$ PUFA can mediate the activity of transcription factors such as nuclear factor κB (NF-κB) [10–12], which plays a key role in regulating the expression of more than 160 genes, including TNF-α, IL-1β and IL-6 [13,14]. Thus, in order to assess the mechanisms underlying the modulation of human THP-1 macrophage cytokine expression and production by LC $n-3$ PUFA, the effects of EPA and DHA on NF-κB activation were investigated.

2. Materials and methods

2.1. Materials

Phosphate-buffered saline tablets were obtained from Oxoid Ltd. (Hampshire, UK). Cell culture media, Hanks balanced salt solution (HBSS), fetal bovine serum (FBS) and deoxyribonuclease I (amplification grade) were obtained from Invitrogen Corporation (Paisley, UK). L-glutamine, penicillin and streptomycin media supplement, phorbol 12-myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS; *Escherichia coli* 055:B5), EPA and DHA were obtained from Sigma-Aldrich (Dorset, UK). TRI Reagent was obtained from Molecular Research Center, Inc. (Ohio, USA). Reagents for reverse transcription were obtained from Promega Corporation (Madison, WI, USA). All other chemicals were obtained from Sigma-Aldrich.

2.2. Cell culture

Human THP-1 monocytes were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). In comparison to other human myeloid cell lines, differentiated THP-1 macrophages behave more like native monocyte-derived macrophages and thus provide a valuable model for studying the regulation of macrophage-specific genes as they relate to the physiological functions displayed by these cells [15]. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin, and incubated at 37°C in 5% CO_2. To induce monocyte–macrophage differentiation, THP-1 cells were cultured in the presence of 100 ng/ml PMA for 72 h. Adherent macrophages were treated with serum-free RPMI 1640 for 24 h. Stocks (100 mM) of EPA and DHA were prepared in DMSO, and cells were treated with 25 or 100 μM EPA and DHA for 48 h. For all experiments, control cells treated with DMSO alone were included (final concentration in the medium, $\leq 0.1\%$). As indicated in the text and figure legends, macrophages were stimulated with LPS for 1 or 24 h, depending on subsequent analysis.

2.3. Toxicology assay

A dose-dependent and time-dependent viability study was completed to assess the effects of a range of EPA and DHA concentrations at a number of time points on macrophage viability. The viability of cells that received EPA and DHA (6.25–100 μM) for 6, 12 and 24 h was quantified spectrophotometrically by MTT assay (Sigma-Aldrich), as per the manufacturer's protocol. Briefly, THP-1 macrophages were cultured with or without the fatty acids for 24 h in 96-well microtiter plates. Control wells containing the medium alone were also included. After a 2-h incubation with MTT, formazan crystals were fully dissolved in MTT solubilization solution, and absorbance was measured at 570 nm. Absorbances were corrected for background readings and were expressed as a percentage of control to normalize data. EPA and DHA concentrations (up to 100 μM) had no significant effect ($P>.1$) on cell viability compared to vehicle control at 6, 12 or 24 h (data not shown).

2.4. Enzyme-linked immunosorbent assay (ELISA)

To allow examination of the effects of EPA and DHA on basal and LPS-stimulated cytokine production, fatty-acid-treated macrophages were cultured in the absence or presence of LPS for 24 h. At the end of culture, supernatants were removed, centrifuged at 3000 rpm for 10 min and stored at −80°C until analysis. TNF-α, IL-1β and IL-6 concentrations in cell culture supernatants were quantified by commercial DuoSet ELISA kits (R&D Systems Europe, Abdingdon, Oxon, UK), according to the manufacturer's instructions.

2.5. Cytokine mRNA analysis

Total RNA was extracted from macrophage monolayers and reverse-transcribed as previously described [16]. Briefly, RNA was extracted using TRI Reagent following the protocol provided. Two micrograms of RNA was DNase-treated and reverse-transcribed in a 25-μl reaction containing 1× reverse transcription buffer [50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol (DTT)], 500 ng of random primers, 10 mM dNTP and 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase.

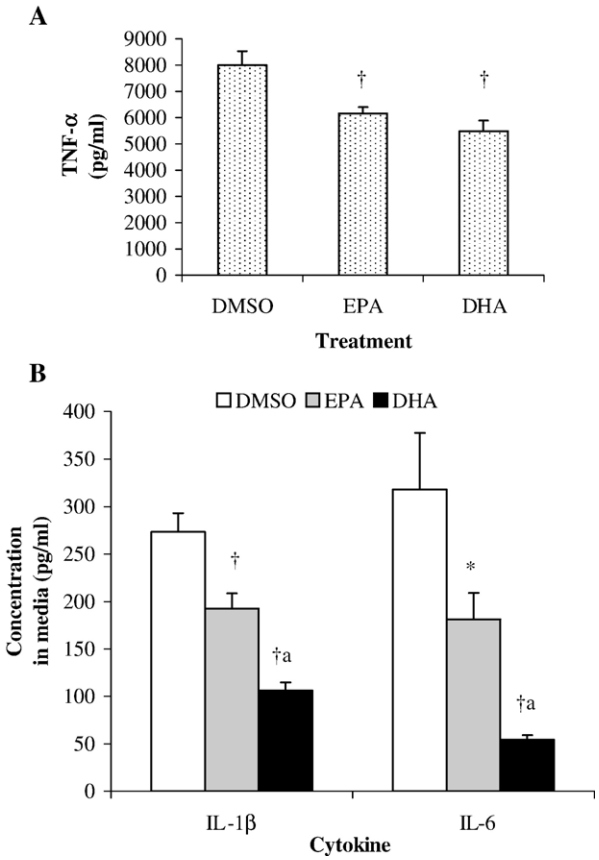

Fig. 1. Effects of 100 μM EPA and DHA on (A) LPS-stimulated TNF-α production and (B) LPS-stimulated IL-1β and IL-6 production. THP-1 macrophages were pretreated for 48 h with vehicle control (DMSO), 100 μM EPA or 100 μM DHA and incubated with 1 μg/ml LPS for a further 24 h. Cytokine concentrations in cell culture supernatants were detected by ELISA. Results represent the mean±S.E.M. of five to six independent experiments. $^{*}P \leq .05$. $^{\dagger}P \leq .001$ relative to DMSO. $^{a}P \leq .05$ relative to EPA.

mRNA expression was quantified by real-time polymerase chain reaction (PCR) on an ABI 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Warrington, UK). All reagents necessary for running a *TaqMan* real-time PCR assay were purchased from Perkin-Elmer Applied Biosystems. *TaqMan* real-time PCR was performed for TNF-α, IL-1β and IL-6 using pre-developed assay reagent kits. Each 25-μl reaction contained 20 ng of cDNA, 2× *TaqMan* Universal PCR Mastermix, forward and reverse primers and *TaqMan* probe. All reactions were performed in duplicate using the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For each sample, results were normalized by dividing the amount of target gene by the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were expressed relative to vehicle control treatment (DMSO).

2.6. Nuclear and cytoplasmic extract preparation

THP-1 macrophages cultured with and without fatty acids were incubated in the absence or presence of 1 μg/ml LPS for 1 h. Cell culture supernatants were then removed, and cell monolayers were washed twice with ice-cold HEPES-buffered HBSS. All buffers for extraction were prepared immediately prior to use and kept ice-cold, and all steps were carried out on ice [17]. Cells were lysed in Buffer A [10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM DTT]. Following centrifugation at 10,000 rpm for 10 min at 4°C, the resulting pellet was resuspended in Buffer A containing 0.1% vol/vol Nonidet P-40. The lysate was incubated on ice for 10 min and centrifuged at 10,000 rpm for 10 min at 4°C.

The resulting supernatant was removed and stored at −80°C as cytoplasmic extract. To prepare nuclear extracts, the pellet was resuspended in Buffer C (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol and 0.5 mM PMSF), incubated on ice for at least 15 min with repeated vortexing and centrifuged at 10,000 rpm for 10 min at 4°C. To the supernatant, Buffer D (10 mM HEPES pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT and 0.5 mM PMSF) was added, and samples were mixed well. The concentration of protein in nuclear and cytoplasmic samples was quantified by Bradford assay (Bio-Rad Laboratories, Inc., California, USA).

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts containing 4 μg of protein were incubated for 30 min at room temperature with 10,000 cpm [^{32}P]-labeled oligonucleotide probe (Perkin-Elmer Life Sciences, Inc., Massachusetts, USA), 2 μg of poly dI–dC (Amersham-Pharmacia, UK), 100 mM Tris pH 7.5, 500 mM NaCl, 40% glycerol (wt/vol), 5 mM EDTA, 5 mM DTT and 1 mg/ml nuclease-free bovine serum albumin. The probes used in EMSA contained the consensus-binding sequence for NF-κB 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, which had been previously labeled with [^{32}P]ATP by T4

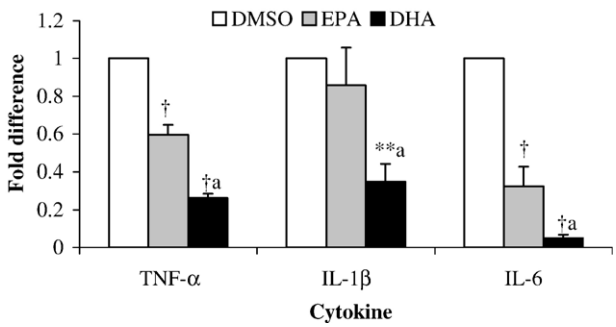

Fig. 2. Effects of 100 μM EPA and DHA on TNF-α, IL-1β and IL-6 mRNA levels in LPS-stimulated macrophage. THP-1 macrophages were pretreated for 48 h with vehicle control (DMSO), 100 μM EPA or 100 μM DHA and incubated with 1 μg/ml LPS for a further 24 h. mRNA expression was analyzed by *TaqMan* real-time PCR. mRNA levels of TNF-α, IL-1β and IL-6 were normalized to GAPDH and expressed relative to DMSO. Results represent the mean±S.E.M. of five to six independent experiments. $^{**}P \leq .01$. $^{\dagger}P \leq .001$ relative to DMSO. $^{a}P \leq .05$ relative to EPA.

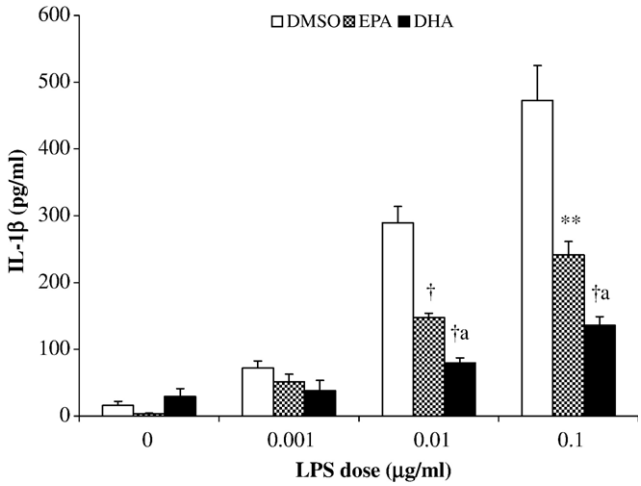

Fig. 3. LPS-induced IL-1β secretion from THP-1 macrophages. Macrophages were pretreated for 48 h with vehicle control (DMSO), 25 μM EPA or 25 μM DHA and stimulated with 0, 0.001, 0.01 and 0.1 μg/ml LPS for 24 h. IL-1β concentrations in cell culture supernatants were detected by ELISA. Results represent the mean±S.E.M. of six independent experiments. $^{**}P \leq .01$. $^{\dagger}P \leq .001$ relative to DMSO. $^{a}P \leq .05$ relative to EPA.

polynucleotide kinase (Promega). DNA–protein complexes were resolved on nondenaturing 4% (vol/vol) polyacrylamide gels. The gels were dried and autoradiographed. After developing, relative band intensities were quantified using GeneSnap Acquisition and GeneTools Analysis Software (GeneGenesis Gel Documentation Analysis System; Syngene).

2.8. Western blot analysis

Fifteen micrograms of nuclear extracts or 75 μg of cytoplasmic protein extracts was precipitated in excess ice-cold acetone and resuspended in sample buffer [62.5 mM Tris–HCl pH 6.8, 10% vol/vol glycerol, 2% wt/vol sodium dodecyl sulfate (SDS), 5% vol/vol β-mercaptoethanol and 0.05% wt/vol bromophenol blue] and denatured by boiling at 100°C for 5 min. The proteins in the nuclear and cytoplasmic extracts were separated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred to a 0.45-μm Biotrace polyvinylidene fluoride transfer membrane (Pall Corporation, Florida, USA). Cytoplasmic and nuclear p65 and IκBα were detected following a 1:1000 dilution of primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). p65 blots were incubated in a 1:1000 dilution of secondary antibody, and IκBα blots were incubated with secondary antibody at a 1:2000 dilution (polyclonal goat antimouse IgG-peroxidase-conjugated immunoglobulins; Sigma-Aldrich). Antigens were detected by an enhanced chemiluminescence substrate reaction (Supersignal West Pico Chemiluminescent Substrate; Pierce, Illinois, USA). Quantification of protein bands was performed using GeneSnap Acquisition and GeneTools Analysis Software (GeneGenesis Gel Documentation Analysis System; Syngene).

2.9. Statistical analysis

Statistical analysis was performed with DataDesk 6.0 (Data Description, Inc., New York, USA). Multiple comparisons were performed by one-way analysis of variance (ANOVA). Individual differences were subsequently tested by Fisher's least significance difference test after a demonstration of significant intergroup differences by ANOVA. In some cases, independent *t* tests were employed, as indicated in the figure legends. A statistical probability of $P \leq .05$ was considered statistically significant.

3. Results

3.1. Anti-inflammatory effect of DHA is greater than that of EPA

The effects of EPA and DHA on LPS-stimulated TNF-α, IL-1β and IL-6 production in THP-1 macrophages are presented in Fig. 1A and B. EPA ($P<.02$), and most especially DHA ($P<.0001$), significantly reduced LPS-induced TNF-α, IL-1β and IL-6 production relative to vehicle control cells. The inhibitory effect of DHA on LPS-induced IL-1β and IL-6 production was significantly greater than that of EPA ($P<.002$), but this was not the case for TNF-α production.

Real-time PCR analysis confirmed that both EPA and DHA reduced LPS-stimulated THP-1 cytokine mRNA expression (Fig. 2). It is interesting to note that, for each cytokine, the anti-inflammatory effect of DHA was significantly more potent than that of EPA ($P<.01$). In LPS-stimulated macrophages, both EPA and DHA significantly reduced TNF-α mRNA expression relative to control cells ($P<.0001$). Only DHA significantly reduced IL-1β mRNA expression relative to control cells ($P<.003$). Both EPA and DHA significantly reduced IL-6 mRNA levels relative to control cells ($P<.0001$).

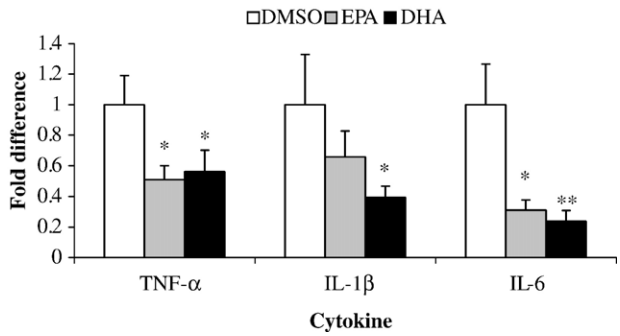

Fig. 4. Effects of 25 μM EPA and DHA on TNF-α, IL-1β and IL-6 mRNA levels in LPS-stimulated macrophages. THP-1 macrophages were pretreated for 48 h with vehicle control (DMSO), 25 μM EPA or 25 μM DHA and incubated with or without 0.1 μg/ml LPS for a further 24 h. mRNA expression was analyzed by *TaqMan* real-time PCR. mRNA levels of TNF-α, IL-1β and IL-6 were normalized to GAPDH and expressed relative to DMSO. Results represent the mean±S.E.M. of four to six independent experiments. $^{*}P \leq .05$ $^{**}P \leq .01$ relative to DMSO.

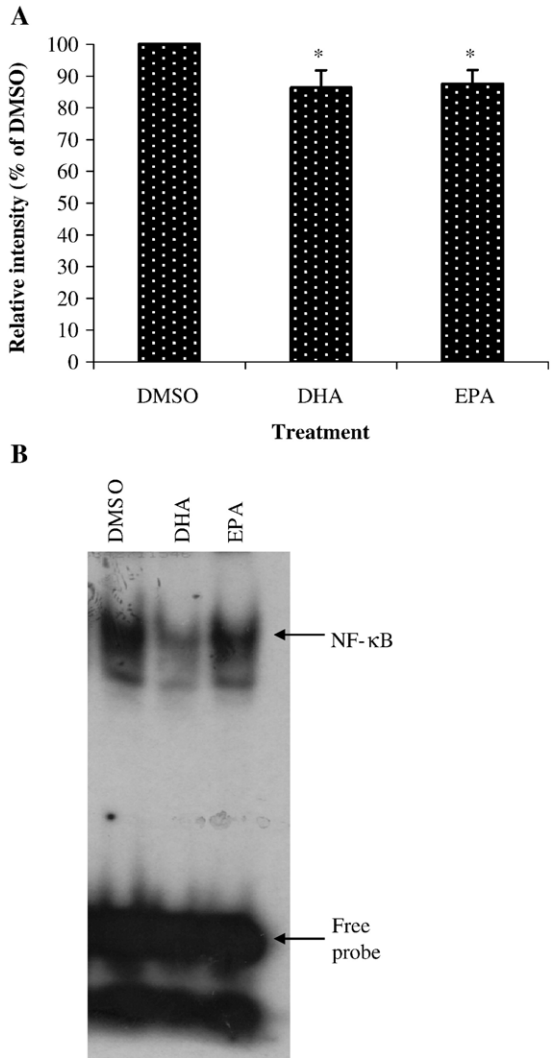

Fig. 5. EPA and DHA inhibit LPS-induced NF-κB/DNA binding activity. THP-1 macrophages were pretreated for 48 h with vehicle control (DMSO), 100 μM EPA and 100 μM DHA and incubated with 1 μg/ml LPS for 1 h. Nuclear extracts were prepared and analyzed by EMSA. (A) Relative band intensity was quantified by densitometry, and data expressed relative to vehicle control cells (DMSO; 100%) are presented as the mean±S.E.M. of three to four independent experiments. (B) A representative EMSA. *P<.05 relative to DMSO (independent t test).

3.2. Dose–response effect of LPS induced cytokine production in the presence of low doses of EPA and DHA

Given the apparent difference in the relative efficacy of EPA and DHA at a relatively high dose (100 μM), we assessed whether this effect was also visible at a lower $n-3$ PUFA concentration (25 μM). Furthermore, because LPS is a critical factor that affects the nature of the cytokine response in vitro, we determined the effects of $n-3$ PUFA on pro-inflammatory cytokine production in response to a range of LPS concentrations (0–0.1 μg/ml). Fig. 3 presents the dose–response effect of LPS on IL-1β secretion in THP-1 macrophages cultured in the presence of 25 μM EPA and DHA. As anticipated, THP-1 macrophage IL-1β production increased significantly in an LPS concentration-dependent fashion (P<.0001). A similar LPS dose–response effect was demonstrated for TNF-α and IL-6 (data not shown). Interestingly, Fig. 3 also shows that 25 μM EPA, and most especially DHA, significantly down-regulated THP-1 macrophage IL-1β production when stimulated with >0.01 μg/ml LPS (P<.0001). The inhibitory effect of DHA on LPS-induced IL-1β production was significantly greater than that seen with EPA in THP-1 macrophages stimulated with both 0.01 and 0.1 μg/ml LPS (P<.01 and P<.04, respectively). DHA also significantly reduced IL-6 secretion to a greater extent than control and EPA-treated cells in response to 0.01 and 0.1 μg/ml LPS (P<.003 and P<.003, respectively) (data not shown), but this was not the case for TNF-α.

In order to assess whether this effect also held true at the transcriptional level, we examined the effects of 25 μM EPA and DHA on LPS-stimulated THP-1 macrophage TNF-α, IL-1β and IL-6 mRNA expression (Fig. 4). Although both $n-3$ PUFA significantly reduced cytokine expression, it was only in the case of IL-1β mRNA expression that DHA appeared to be more efficacious than EPA (P<.05). TNF-α and IL-6 mRNA levels were significantly reduced by both EPA and DHA relative to control cells (P<.05 and P<.03); however, at the lower dose, there was no apparent difference in efficacy between $n-3$ PUFA.

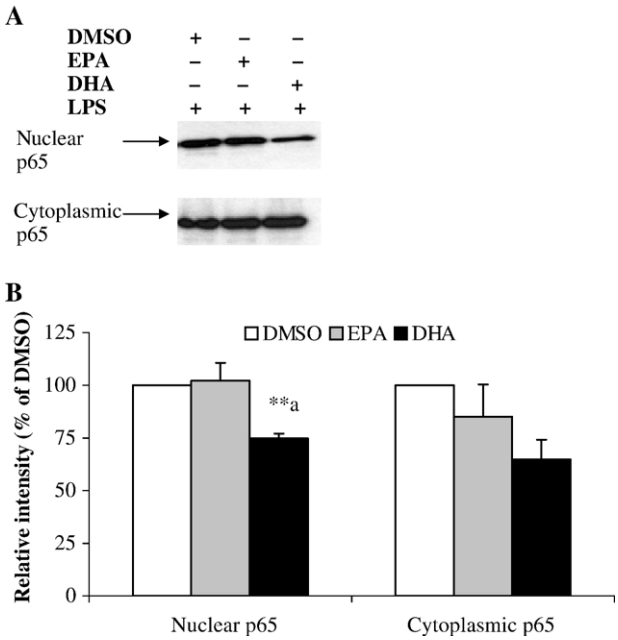

Fig. 6. The effects of EPA and DHA on nuclear and cytoplasmic p65 levels in LPS-stimulated macrophages. THP-1 macrophages were treated for 48 h with vehicle control (DMSO), 100 μM EPA or 100 μM DHA. Macrophages were then incubated for 1 h in the presence of 1 μg/ml LPS. Nuclear and cytoplasmic extracts were prepared for SDS-PAGE and immunoblotting for p65. (A) Representative blot. (B) Relative protein levels were quantified by densitometry, and data expressed relative to the amount of p65 in vehicle control cells (DMSO; 100%) are presented as the mean±S.E.M. of four to five independent experiments. **P<.01 relative to DMSO. [a]P ≤.05 relative to EPA.

3.3. DHA down-regulated components of the NF-κB transcriptional system more than did EPA

Relative to vehicle control-treated cells, both DHA (−14%) and EPA (−13%) significantly decreased NF-κB/DNA binding in macrophages stimulated with LPS for 1 h ($P<.05$) (Fig. 5). Nuclear translocation of NF-κB is required for transcriptional activation; therefore, the effects of EPA and DHA on nuclear and cytoplasmic p65 levels were also examined in these cells (Fig. 6). In support of previous findings, we found that nuclear p65 levels were significantly reduced by DHA compared to both vehicle control cells ($P<.004$) and EPA-treated cells ($P<.003$). Macrophage cytoplasmic p65 expression was also reduced more by DHA following LPS stimulation, but this change did not reach statistical significance ($P=.08$). In contrast to DHA, EPA had no significant effect on nuclear or cytoplasmic p65 level. Fatty acids also had no significant effect on the abundance of nuclear and cytoplasmic p65 in resting THP-1 macrophages (data not shown).

Given that IκBα serves to inhibit NF-κB transcriptional activity, we examined the individual effects of EPA and DHA on nuclear and cytoplasmic IκBα levels in THP-1 macrophages stimulated with LPS for 1 h (Fig. 7). DHA significantly increased cytoplasmic IκBα expression in LPS-stimulated THP-1 macrophages compared to both control ($P<.01$) and EPA-treated ($P<.04$) cells. Although EPA induced a marginal increase in cytoplasmic IκBα levels (35%) in LPS-stimulated THP-1 macrophages, this effect was not statistically significant. Neither EPA nor DHA had a significant effect on IκBα expression in resting macrophage nuclear and cytoplasmic extracts (data not shown).

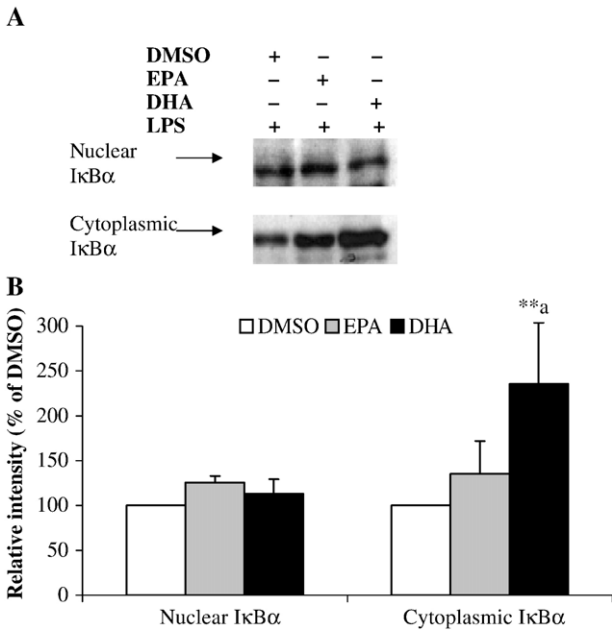

Fig. 7. The effects of EPA and DHA on nuclear and cytoplasmic IκBα levels in LPS-stimulated macrophages. THP-1 macrophages were treated for 48 h with vehicle control (DMSO), 100 μM EPA or 100 μM DHA. Macrophages were then incubated for 1 h in the presence of 1 μg/ml LPS. Nuclear and cytoplasmic extracts were prepared for SDS-PAGE and immunoblotting for IκBα. (A) Representative blot. (B) Relative protein levels were quantified by densitometry, and data expressed relative to the amount of IκBα in vehicle control cells (DMSO; 100%) are presented as the mean±S.E.M. of two to five independent experiments. $^{**}P<.01$ relative to DMSO. $^{a}P\leq.05$ relative to EPA.

4. Discussion

This study demonstrates that DHA suppressed the mRNA expression and production of several pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, in LPS-stimulated THP-1 monocyte-derived macrophages to a greater extent than did EPA. Our findings also suggest that DHA has greater effects on the protein levels of the components of the NF-κB transcriptional system. Although both LC $n-3$ PUFA were associated with reduced NF-κB/DNA binding, DHA lowered nuclear p65 expression and increased cytoplasmic IκBα expression to a significantly greater extent in LPS-stimulated THP-1 macrophages. These observations were based on relatively high $n-3$ PUFA doses (100 μM), which we extended to confirm that a lower DHA concentration (25 μM) significantly reduced THP-1 macrophage IL-1β and IL-6 secretion more effectively than did EPA over a range of LPS concentrations. Our findings concur and extend the findings of several human studies, which reported that fish oil reduced TNF-α, IL-1β and IL-6 production in LPS-stimulated mononuclear cells [7,8,18,19]. It should be noted that some human studies have shown no effect of fish oils on pro-inflammatory cytokines [9,20–22]. Our findings suggest that apparent discrepancies between human supplementation studies could be ascribed to the differential effects of LC $n-3$ PUFA on markers of inflammatory response.

Studies of the effects of $n-3$ PUFA on cytokine production in pure human macrophage populations are limited. Our findings largely concur and extend the findings of few in vitro macrophage studies that determined either the combined or the individual effects of EPA and DHA. Novak et al. [23] and Babcock et al. [24] demonstrated a 46% reduction in TNF-α production by LPS-stimulated (1 μg/ml) murine RAW 264.7 macrophages that were cultured for 4 h with Omegaven (a commercial lipid emulsion containing 1.25–2.82 g of EPA and 1.44–3.09 g of DHA per 100 ml), compared to control cells. Chu et al. [25] reported significantly reduced TNF-α and IL-1β secretion from LPS-stimulated human THP-1 monocytes pretreated for 72 h with 10 μM EPA and 10 μM DHA. Similarly, Zhao et al. [12] reported significantly reduced LPS-stimulated TNF-α production from human THP-1 monocytes pretreated for 24 h with both 60 μM EPA and 60 μM DHA. Similarly, in a recent publication, Zhao et al. [26] also reported that 100 μM DHA significantly decreased the production and expression of TNF-α, IL-1β and IL-6 in LPS-stimulated (1 μg/ml) THP-1 monocytes. Lo et al. [10] observed reduced TNF-α production by LPS-stimulated

murine RAW macrophage treated with 114 μM EPA for 24 h. Mice fed a fish oil diet for 8 weeks showed a significantly reduced plasma IL-6 response to the trichothecene mycotoxin deoxynivalenol (DON) compared with mice fed a control corn oil diet [27]. In addition, the authors demonstrated that EPA and DHA reduced IL-6 production in LPS-stimulated and DON-stimulated RAW macrophages [27].

Cytokines have a short half-life (their secretion depends on new protein synthesis), and the elaboration of cytokine response to inflammatory stimuli is ultimately regulated by the transcription rates of cytokine genes [28]. We demonstrated that both high (100 μM) and low (25 μM) EPA and DHA concentrations markedly reduced LPS-stimulated TNF-α, IL-1β and IL-6 mRNA expression, in accordance with their effects on cytokine production. However, further work is required to establish the optimal concentrations of fatty acids that are of benefit in this pro-inflammatory model, although it is possible that these concentrations will vary between genes, proteins and other end points of measurements. At the higher dose, DHA repressed cytokine IL-1β and IL-6 mRNA expression more effectively than did EPA. In the case of IL-1β, this apparent differential efficacy was reproduced at the lower DHA concentration (25 μM). The reduction of TNF-α mRNA by the LC $n-3$ PUFA corresponds with the findings of Lo et al. [10] and Novak et al. [23], who observed a reduction in TNF-α mRNA in LPS-stimulated murine RAW macrophages treated with 114 μM EPA and with a fish-oil-based lipid emulsion Omegaven, respectively. NF-κB plays key roles in regulating cytokine gene transcription. The most abundant form of NF-κB is the p65/p50 heterodimer, in which the p65 subunit contains the transcriptional activation domain. In resting cells, NF-κB is sequestered in the cytoplasm in an inactive state bound to IκBα [13]. Activation by LPS requires sequential phosphorylation of IκBα, ubiquitination and degradation by the proteasome, followed by translocation of NF-κB to the nucleus [13]. Both EPA and DHA decreased NF-κB/DNA binding in THP-1 macrophage nuclear extracts. However, Western blot analysis suggested that DHA had greater effects on the components of the NF-κB transcriptional complex. In LPS-stimulated macrophages, DHA decreased nuclear p65 expression and increased cytoplasmic IκBα levels. Overall, in conjunction with cytokine data, our findings suggest that DHA may be a more potent anti-inflammatory LC $n-3$ PUFA than EPA.

Our findings agree with a limited number of studies, which suggest that $n-3$ PUFA down-regulate NF-κB activity, both in animal and other cell models. Xi et al. [29] found that mice fed fish oil had reduced NF-κB activity in LPS-stimulated spleen cells. In addition, Novak et al. [23] reported reduced LPS-stimulated NF-κB activity and IκB phosphorylation in murine RAW macrophages treated with $n-3$ PUFA lipid emulsion. In contrast, Lo et al. [10] demonstrated reduced p65/p50 expression in RAW macrophages incubated in EPA-rich media. In addition, EPA reduced LPS-induced NF-κB activation in human THP-1 monocytes [12]. The DHA-specific effects reported in our study agree with Komatsu et al. [11], who reported that DHA (60 μM) inhibited NF-κB activity in RAW macrophages stimulated with interferon-γ and LPS.

Overall, our findings indicate that the inverse relationship between LC $n-3$ PUFA and inflammation may at least be due partly to the greater ability of DHA to modulate macrophage cytokine secretion. The effects of DHA on LPS-induced responses were, in general, more potent than those for EPA. It is therefore possible that EPA and DHA have divergent mechanisms of action. Few comparisons between EPA and DHA have been previously made in vitro. Chu et al. [25] observed no difference between EPA and DHA on THP-1 monocyte TNF-α and IL-1β production. Similarly, there was no apparent difference between the effects of EPA and DHA on IL-6 production in murine macrophages [27]. In contrast, Khalfoun et al. [30] reported a more potent reduction in IL-6 by stimulated lymphocytes with EPA, compared to reduction in IL-6 by stimulated lymphocytes with DHA. Thus, our results are the first to demonstrate a more potent reduction in stimulated macrophage production of cytokines by DHA than by EPA in vitro.

The reasons why the anti-inflammatory effects of DHA are more potent than those of EPA are unclear, but differences between the activities of EPA and DHA may account for such observed effect. For example, EPA can compete with arachidonic acid (AA), act as a substrate for cyclooxygenase and lipoxygenase enzymes and be converted to eicosanoids, while DHA cannot. Consequently, changes in levels of pro-inflammatory and anti-inflammatory eicosanoids may be involved. Lokesh et al. [31] reported that DHA-enriched murine peritoneal macrophages produced less of the pro-inflammatory eicosanoid leukotriene B_4 than EPA-enriched cells; however, cytokine production was not measured. Although their action in antagonizing AA metabolism and subsequent eicosanoid and prostaglandin production is a key anti-inflammatory action of $n-3$ PUFA, current evidence suggests that the effects of LC $n-3$ PUFA on pro-inflammatory cytokine production might occur downstream of altered eicosanoid synthesis or may indeed be independent of this activity. There is now a vast amount of evidence, including the findings presented here, to suggest that some of the anti-inflammatory effects of LC $n-3$ PUFA may be exerted at the level of altered gene expression via direct or indirect actions on intracellular signaling pathways, which lead to the activation of transcription factors such as NF-κB. However, further work is required to delineate these actions.

Although NF-κB appears to play a role in the apparent anti-inflammatory effect ascribed particularly to DHA, the role of other transcription factors such as AP-1 must also be considered. LC $n-3$ PUFA have been shown to reduce the activation of mitogen-activated protein kinase cascade and AP-1 activity in murine RAW macrophages [27,32,33]. In addition, factors that determine posttranscriptional mRNA

processing and stability, translation and posttranslational modifications require further investigation. It is also possible that the fatty acids may alter the cellular redox status, which could in turn affect NF-κB activity, the full nature of which needs further investigation. In a study by Komatsu et al. [11], DHA was a more potent inhibitor of NF-κB-mediated nitric oxide compared to EPA, and DHA was shown to reduce oxidative stress and to increase levels of the antioxidant glutathione (GSH). Therefore, DHA may inhibit NF-κB activation by triggering antioxidant systems such as GSH. Chu et al. [25] suggested that *n*−3 PUFA are able to block LPS transmembrane signaling that mediates a variety of significant antagonisms against LPS action, including decreased TNF-α and IL-1β production. Thus, there are many aspects of the potential anti-inflammatory effects of EPA and DHA metabolism that remain unclear.

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References

[1] Yaqoob P, Calder PC. *n*−3 polyunsaturated fatty acids and inflammation in the arterial wall. Eur J Med Res 2003;8:337–54.
[2] Calder PC, Zurier RB. Polyunsaturated fatty acids and rheumatoid arthritis. Curr Opin Clin Nutr Metab Care 2001;4:115–21.
[3] Alexander JW. Immunonutrition: the role of ω-3 fatty acids. Nutrition 1998;14:627–33.
[4] Billiar TR, Bankey PE, Svingen BA, Curran RD, West MA, Holman RT, et al. Fatty acid intake and Kupffer cell function: fish oil alters eicosanoid and monokine production to endotoxin stimulation. Surgery 1988;104:343–9.
[5] Renier G, Skamene E, DeSanctis J, Radzioch D. Dietary *n*−3 polyunsaturated fatty acids prevent the development of atherosclerotic lesions in mice: modulation of macrophage secretory activities. Arterioscler Thromb 1993;13:1515–24.
[6] Yaqoob P, Calder PC. Effects of dietary lipid manipulation upon inflammatory mediator production by murine macrophages. Cell Immunol 1995;163:120–8.
[7] Meydani SN, Endres S, Woods MM, Goldin BR, Soo C, Morrill-Labrode A, et al. Oral (*n*−3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. J Nutr 1991;121:547–55.
[8] Caughey GE, Mantzioris E, Gibson RA, Cleland LG, James MJ. The effect on human tumor necrosis factor α and interleukin 1β production of diets enriched in *n*−3 fatty acids from vegetable oil or fish oil. Am J Clin Nutr 1996;63:116–22.
[9] Kew S, Mesa MD, Tricon S, Buckley R, Minihane AM, Yaqoob P. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. Am J Clin Nutr 2004;79:674–81.
[10] Lo C-J, Chiu KC, Fu M, Lo R, Helton S. Fish oil decreases macrophage tumor necrosis factor gene transcription by altering the NFκB activity. J Surg Res 1999;82:216–21.
[11] Komatsu W, Ishihara K, Murata M, Saito H, Shinohara K. Docosahexaenoic acid suppresses nitric oxide production and inducible nitric oxide synthase expression in interferon-γ plus lipopolysaccharide-stimulated murine macrophages by inhibiting the oxidative stress. Free Radic Biol Med 2003;34:1006–16.
[12] Zhao Y, Joshi-Barve S, Barve S, Chen LH. Eicosapentaenoic acid prevents LPS-induced TNF-α expression by preventing NF-κB activation. J Am Coll Nutr 2004;23:71–8.
[13] Ghosh S, May MJ, Kopp EB. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 1998;16:225–60.
[14] Kopp E, Ghosh S. NF-κB and rel proteins in innate immunity. Adv Immunol 1995;58:1–27.
[15] Auwerx J. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. Experientia 1991;47:22–31.
[16] Weldon S, Mitchell S, Kelleher K, Gibney MJ, Roche HM. Conjugated linoleic acid and atherosclerosis: no effect on molecular markers of cholesterol homeostasis in THP-1 macrophages. Atherosclerosis 2004;174:261–73.
[17] Osborn L, Kunkel S, Nabel GJ. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. Proc Natl Acad Sci U S A 1989;86:2336–40.
[18] Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, van der Meer JWM, et al. The effect of dietary supplementation with *n*−3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med 1989; 320:265–71.
[19] Gallai V, Sarchielli P, Trequattrini A, Franceschini M, Floridi A, Firenze C, et al. Cytokine secretion and eicosanoid production in the peripheral blood mononuclear cells of MS patients undergoing dietary supplementation with *n*−3 polyunsaturated fatty acids. J Neuroimmunol 1995;56:143–53.
[20] Molvig J, Pociot F, Worsaae H, Wogensen LD, Baek L, Christensen P, et al. Dietary supplementation with omega-3-polyunsaturated fatty acids decreases mononuclear cell proliferation and interleukin-1 beta content but not monokine secretion in healthy and insulin-dependent diabetic individuals. Scand J Immunol 1991; 34:399–410.
[21] Schmidt EB, Varming K, Moller JM, Bulow Pedersen I, Madsen P, Dyerberg J. No effect of a very low dose of *n*−3 fatty acids on monocyte function in healthy humans. Scand J Clin Lab Invest 1996;56:87–92.
[22] Blok JE, Deslypere JP, Demacker PNM, van der Ven-Jongekrijg J, Hectors MPC, van der Meer JMW, et al. Pro- and anti-inflammatory cytokines in healthy volunteers fed various doses of fish oil for 1 year. Eur J Clin Invest 1997;27:1003–8.
[23] Novak TE, Babcock TA, Jho DH, Helton WS, Espat NJ. NF-kappa B inhibition by omega-3 fatty acids modulates LPS-stimulated macrophage TNF-alpha transcription. Am J Physiol Lung Cell Mol Physiol 2003;284:L84–9.
[24] Babcock TA, Helton WS, Hong D, Espat NJ. Omega-3 fatty acid lipid emulsion reduced LPS-stimulated macrophage TNF-α production. Surg Infect 2002;3:145–9.
[25] Chu AJ, Walton MA, Prasad JK, Seto A. Blockade by polyunsaturated *n*−3 fatty acids of endotoxin-induced monocytic tissue factor activation is mediated by depressed receptor expression in THP-1 cells. J Surg Res 1999;87:217–24.
[26] Zhao G, Etherton TD, Martin KR, Vanden Heuvel JP, Gillies PJ, West SG, et al. Anti-inflammatory effects of polyunsaturated fatty acids in THP-1 cells. Biochem Biophys Res Commun 2005;336: 909–17.
[27] Moon Y, Pestka JJ. Deoxynivalenol-induced mitogen-activated protein kinase phosphorylation and IL-6 expression in mice suppressed by fish oil. J Nutr Biochem 2003;14:717–26.
[28] Blackwell TS, Christman JW. The role of nuclear factor-κB in cytokine gene regulation. Am J Respir Cell Mol Biol 1997;17: 3–9.
[29] Xi S, Cohen D, Barve S, Chen LH. Fish oil suppressed cytokines and nuclear factor-kappaB induced murine AIDS virus infection. Nutr Res 2001;21:865–78.

[30] Khalfoun B, Thibault F, Watier H, Bardos P, Lebranchu Y. Docosahexaenoic acid and eicosapentaenoic acids inhibit in vitro human endothelial cell production of interleukin-6. Adv Exp Med Biol 1997;400B:589–97.

[31] Lokesh BR, German B, Kinsella JE. Differential effects of docosahexaenoic acid and eicosapentaenoic acid on suppression of lipoxygenase pathway in peritoneal macrophages. Biochim Biophys Acta 1988;958:99–107.

[32] Lo C-J, Chiu KC, Fu M, Chu A, Helton S. Fish oil modulates macrophage P44/P42 mitogen-activated protein kinase activity induced by lipopolysaccharide. JPEN J Parenter Enteral Nutr 2000;24:159–63.

[33] Babcock TA, Kurland A, Jelton WS, Rahman A, Anwar KN, Espat NJ. Inhibition of activator protein-1 transcription factor activation by ω-3 fatty aid modulation of mitogen-activated protein kinase signalling kinases. JPEN J Parenter Enteral Nutr 2003;27:176–81.