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Research Articles

Eicosapentaenoic acid prevents lipopolysaccharide-stimulated DNA binding of activator protein-1 and c-Jun N-terminal kinase activity

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Abstract

Activator protein-1 (AP-1) is a transcription factor that plays an important role in regulating the expression of proinflammatory cytokines including tumor necrosis factor-α (TNF-α). Many studies have shown that fish oil supplementation inhibits TNF-α production in mice and humans; however, the mechanisms remain unclear. In this study, the effects of eicosapentaenoic acid (EPA), a major n-3 fatty acid in fish oil, on lipopolysaccharide (LPS)-stimulated activation of AP-1 were investigated in human monocytic THP-1 cells. The results demonstrated that AP-1 DNA binding activity stimulated by LPS was suppressed by preincubating cells with EPA. Lipopolysaccharide-stimulated increase of c-Jun and c-Fos protein levels was also attenuated by EPA pretreatment. In addition, EPA pretreatment decreased LPS-induced c-Jun phosphorylation and c-Jun N-terminal kinase (JNK) activation. The results suggest that suppression of TNF-α expression by EPA may be partly mediated by its inhibitory effect on AP-1 activation.

Keywords: Eicosapentaenoic acid; LPS; AP-1; c-Jun; JNK; TNF-α

1. Introduction

Tumor necrosis factor-α (TNF-α) is a principal mediator of inflammation. It is involved in the pathogenesis of many human diseases including rheumatoid arthritis, AIDS, cancer cachexia, inflammatory bowel disease and multiple sclerosis [1,2]. The primary sources of TNF-α production are monocytes and macrophages [3]. Lipopolysaccharide (LPS), a surface component of gram-negative bacteria, is one of the strongest stimuli that induce TNF-α production [4]. Overreaction to LPS leads to tissue injury and shock, and the adverse effects of LPS are partly mediated by TNF-α [5,6]. Lipopolysaccharide stimulation of monocytes activates several intracellular signaling pathways, including mitogen-activated kinases (MAPK) and IκB kinase [7–11]. These signaling pathways activate a variety of transcription factors such as NF-κB and activator protein-1 (AP-1), leading to the activation of TNF-α gene expression [12].

Activator protein-1 is a complex composed of proteins of Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) families [13,14]. Activator protein-1 binding sites at the promoter region of TNF-α gene are suggested to be critical for TNF-α expression [12]. The activity of AP-1 can be regulated through changing the composition and abundance of AP-1 complexes, and by modifying its components via specific protein kinases [13,15,16]. Most of the genes that encode AP-1 components such as *fos* and *jun* are rapidly induced in response to extracellular stimuli [13]. In addition, the activities of both preexisting and newly synthesized AP-1 components are modulated through phosphorylation [15,16]. The activation domain of c-Jun is regulated by c-Jun N-terminal kinase (JNK) family of mitogen-activated protein (MAP) kinases. c-Jun N-terminal kinase phosphorylates c-Jun on NH_2-terminal Ser-63 and Ser-73 and stimulates its transcriptional activity [16–18].

Eicosapentaenoic acid (EPA) is a n-3 polyunsaturated fatty acid mainly found in fish oil. Numerous studies have shown that supplementation of fish oil or n-3 fatty acids such as EPA to humans and mice decreases TNF-α production, yet the precise mechanisms remain unclear [19–22]. Eicosapentaenoic acid suppresses LPS-induced TNF-α expression

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in THP-1 cells, a human monocytic cell line that displays many characteristics similar to human monocytes [23,24]. The objectives of this study were to explore the molecular mechanisms by which EPA modulates TNF-α expression, in particular, to examine the effect of EPA on AP-1 activation. We observed that EPA decreased LPS-stimulated AP-1 DNA binding, LPS-induced c-Jun phosphorylation and JNK activity in THP-1 cells. Therefore, the modulation of TNF-α gene expression by EPA may be partly mediated by its effect on AP-1 activation.

2. Materials and methods

2.1. Materials

cis-5,8,11,14,17-EPA, sodium salt and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS), penicillin and streptomycin were purchased from Life Technologies (Grand Island, NY). Antibodies for phosphorylated c-Jun (p-c-Jun), JNK and phosphorylated JNK were purchased from New England Biolab (Beverly, MA). Other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma unless they are stated otherwise.

2.2. Methods

2.2.1. Cell culture

THP-1 cell line was kindly provided by Dr. Shirish Barve, Department of Internal Medicine, University of Kentucky. Cells were maintained in RPMI 1640 medium supplemented with 1% penicillin and streptomycin, 2 mM glutamine, 10% FBS and 2×10^{-5} M 2-mercaptoethanol at 37°C in a 5% CO_2 humid atmosphere. Eicosapentaenoic acid sodium salt was dissolved in RPMI 1640 medium with 5% FBS to make a stock solution. Cells with a density of 1×10^6 were preincubated with 60 μM of EPA for 24 h and then stimulated with 0.2 μg/ml of LPS. The incubation time of EPA was chosen for the maximum incorporation of EPA into cell membrane. Analysis of fatty acid content in the neutral lipids and phospholipids showed a significant increase of EPA content after cells were treated with EPA for 24 h [23]. The viability of the cells was checked with trypan blue exclusion assay. No significant effect of EPA on cell viability was found at the concentrations used in the experiment.

2.2.2. Preparation of total cell lysates and nuclear extracts

For preparing total cell extracts, cells were washed twice with cold phosphate-buffered saline and resuspended in 100 μl of cell lysis buffer containing 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride. After 5 min of incubation on ice, cell lysates were sonicated and centrifuged at 15,000 rpm for 10 min at 4°C. The supernatants were collected and stored at −70°C. For preparing nuclear extracts, cells were washed in cold phosphate-buffered saline, resuspended in 500 μl of cold buffer (10 mM HEPES, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupepstatin, 1 μg/ml pepstatin and 1 μg/ml leucine thiol, 0.1% IgePal CA 630, pH 7.9) and incubated on ice for 30 min. The samples were then mixed and centrifuged at 5000 rpm for 30 min. The pellets were resuspended in 150 μl of cold saline buffer [20 mM HEPES, 50 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 300 mM NaCl, 25% (v/v) glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupepstatin, 1 μg/ml pepstatin and 1 μg/ml leucine thiol, 0.2% IgePal CA 630, pH 7.9] and incubated on ice for 1 h. After centrifuging at 15,000 rpm for 15 min at 4°C, the supernatant containing the nuclear proteins was collected and stored at −70°C.

2.2.3. Western blot analysis

Protein concentration was determined as described by Lowry et al. [25]. Briefly, 1 ml of sample or standard was added to equal volume of alkaline copper solution and stood at room temperature for 10 min. Three milliliters of Folin phenol reagent was added. The samples and standards were heated in a water bath at 50°C for 10 min and cooled at room temperature. The absorbance was read at 540 nm with a spectrophotometer (Shimadzu UV 160U). Fifty micrograms of protein extracts were resolved by SDS-polyacrylamide gel electrophoresis, then transferred electrophoretically to nitrocellulose and subsequently incubated with specific primary antibodies. For detection, the nitrocellulose filter was incubated with a horseradish peroxidase-coupled secondary antibody, followed by an enhanced chemiluminescence substrate reaction using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Equal loading and transferring were examined by staining the membranes with 0.1% Ponceau S. solution.

2.2.4. Electrophoretic mobility shift assay

DNA binding of AP-1 was characterized by electrophoretic mobility shift assay (EMSA) [26]. Nuclear extracts containing 5 to 10 μg of protein were incubated with 0.2 ng of ^{32}P-labeled oligonucleotide probe, 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM EDTA, 4% glycerol (v/v) and 0.08 mg/ml sonicated salmon sperm DNA at room temperature for 30 min. The oligonucleotide probes used in the EMSA contained consensus-binding sequence for AP-1, 5′ -CGC TTG ATG ACT CAG CCG GAA-3′ . DNA–protein complex with a total volume of 20 μl was resolved in a nondenaturing 6% (w/v) polyacrylamide gel and run for 1 h at 200 V in 0.25×TBE (2.5 mM Tris, 2.5 mM H_3BO_3, 2 mM EDTA, pH 8.5). The gel was then dried and autoradiographed using Kodak X-ray film. For competition experiments, unlabeled

probes were added in excess (50×) in the binding buffer. The mutant oligonucleotide, 5′ -CGC TTG ATG ACT *TG*G CCG GAA-3′ , has a "CA"→"TG" substitution in the AP-1 binding motif [27]. Probes were labeled with [γ^{32}P]dATP. The labeled probes were purified using Nick spin column from Amersham Pharmacia Biotech. Antibodies were added in the reaction buffer for supershift assay [28].

2.2.5. JNK kinase assay

c-Jun N-terminal kinase kinase assay was performed using a commercial kit from New England Biolab following the protocols provided. In brief, an N-terminal c-Jun (1–89) fusion protein bound to glutathione sepharose beads was used to selectively "pull down" JNK from cell lysates. The beads were then washed to remove nonspecifically bound proteins and the kinase reaction was carried out in the presence of ATP. Finally, c-Jun phosphorylation was determined by Western blotting using an antibody that specifically recognizes p-c-Jun.

3. Results

3.1. Effects of EPA on LPS-stimulated DNA binding activity of AP-1

DNA binding activity of AP-1 was observed in unstimulated THP-1 cells (Fig. 1A). Lipopolysaccharide stimulation enhanced the DNA binding activity of AP-1, while pretreating cells with EPA apparently prevented the LPS-stimulated AP-1 DNA binding to a great extent (Fig. 1A). The specificity of AP-1 binding was confirmed by the competition assay (Fig. 1B) and the supershift assay (Fig. 1C).

3.2. Effects of EPA on LPS-stimulated c-Jun and c-Fos levels

Since the supershift assay demonstrated that both c-Jun and c-Fos were present in the AP-1 complex, we then examined the effects of EPA pretreatment on the protein levels of c-Jun and c-Fos in LPS-stimulated THP-1 cells. As shown in Fig. 2, basal levels of c-Jun and c-Fos were

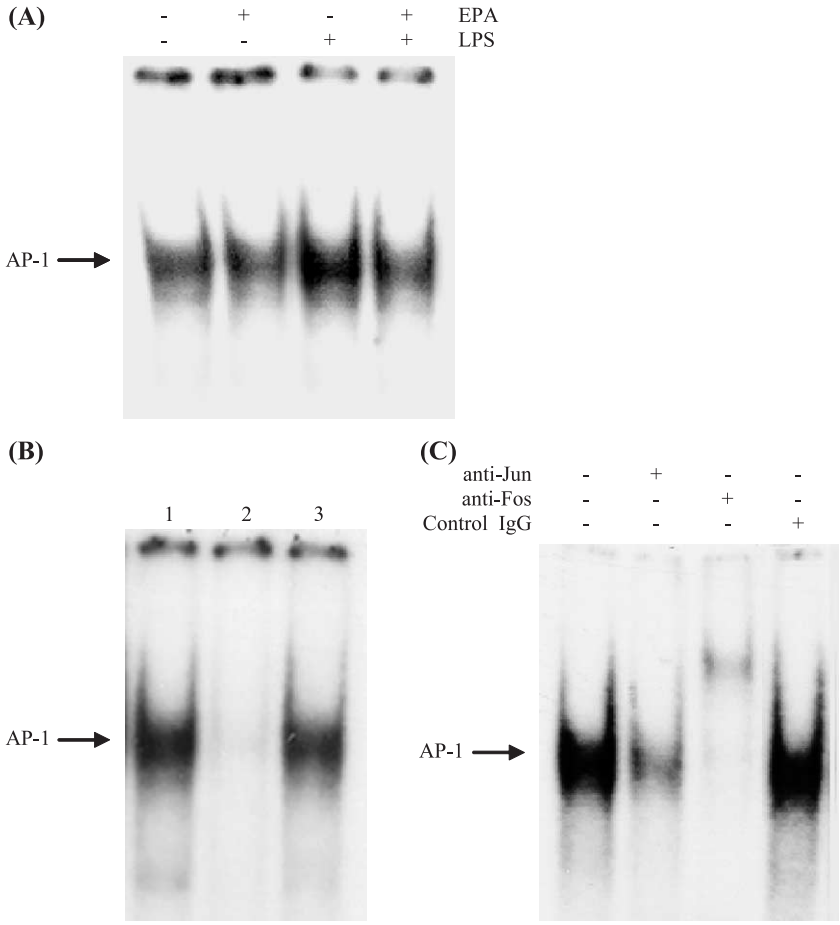

Fig. 1. Effects of EPA on LPS-stimulated DNA binding of AP-1. THP-1 cells preincubated with or without 60 μM of EPA for 24 h were stimulated with 0.2 μg/ml of LPS for 1 h. (A) Nuclear extracts were prepared and analyzed by EMSA. (B) The competition assay was carried out by incubating nuclear extracts prepared from LPS-stimulated THP-1 cells with labeled probes (1), labeled probes plus excess unlabeled probes (2) or labeled probes plus excess mutated oligonucleotide (3). The mutated oligonucleotide has a CA→TG substitution in the AP-1 DNA binding motif as described in Material and methods. (C) For the supershift assay, antibodies of Jun, Fos or control IgG were incubated with the nuclear extracts of LPS-stimulated THP-1 cells and EMSA was performed. Data are representative of three experiments.

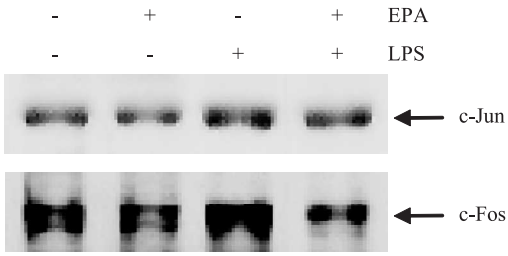

Fig. 2. Effects of EPA on LPS-stimulated c-Jun and c-Fos levels. THP-1 cells preincubated with or without 60 μM of EPA for 24 h were stimulated with 0.2 μg/ml of LPS for 1 h. Protein levels of c-Jun and c-Fos were analyzed by Western blot. Data are representative of two experiments.

observed in the unstimulated THP-1 cells. The amounts of c-Jun and c-Fos were up-regulated by LPS-stimulation. Pretreating cells with EPA partially prevented LPS-stimulation of c-Jun and c-Fos (Fig. 2).

3.3. Effects of EPA on LPS-induced c-Jun phosphorylation and JNK activation

Lipopolysaccharide stimulation activates JNK, which phosphorylates the transcriptional activation domain of c-Jun and greatly enhances the trans-activating potential of c-Jun [16–18]. There was little p-c-Jun in THP-1 cells without LPS stimulation. We observed an increase of p-c-Jun at 30 and 60 min after LPS stimulation, and to a much greater extent, at 60 min (Fig. 3A). Preincubating cells with EPA significantly decreased LPS-induced phosphorylation of c-Jun at both 30 and 60 min after LPS stimulation (Fig. 3A). c-Jun N-terminal kinase activity was then determined by an in vitro kinase assay. The results indicated that LPS-induced JNK activities, as indicated by phosphorylated-GST-c-Jun (p-GST-c-Jun) levels, were also lower in the cells preincubated with EPA (Fig. 3B).

The activity of JNK is mainly regulated by phosphorylation rather than changing the amount of the enzymes.

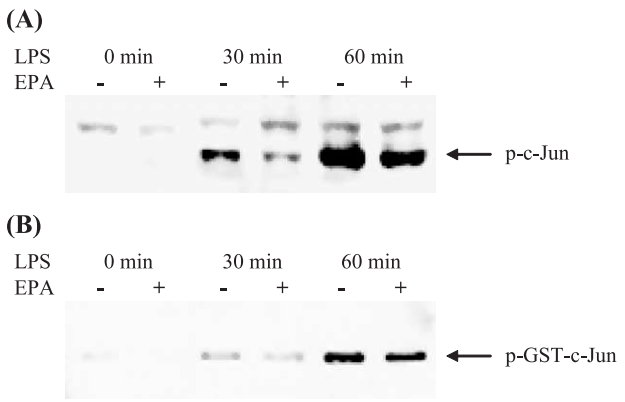

Fig. 3. Effects of EPA on LPS-induced c-Jun phosphorylation. THP-1 cells preincubated with or without 60 μM of EPA for 24 h were stimulated with 0.2 μg/ml of LPS for 0, 30 and 60 min. (A) Protein levels of p-c-Jun were analyzed by Western blot. (B) JNK activity was determined by an in vitro kinase assay using a GST-c-Jun fusion protein as described as in Material and methods. Phosphorylated GST-c-Jun (p-GST-c-Jun). Data are representative of two experiments.

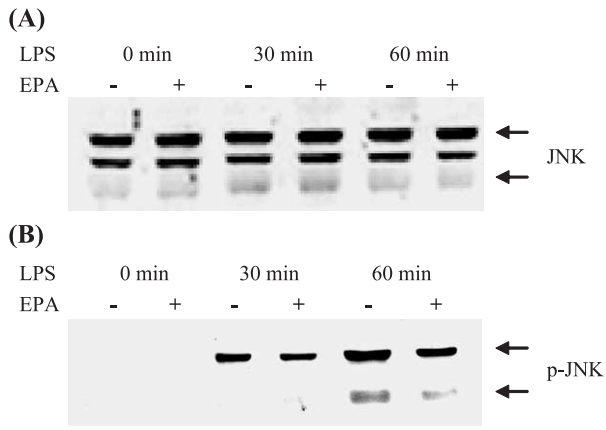

Fig. 4. Effects of EPA on LPS-induced JNK and phosphorylated JNK levels. THP-1 cells preincubated with or without 60 μM of EPA for 24 h were stimulated with 0.2 μg/ml of LPS for 0, 30 and 60 min. Protein levels of (A) JNK and (B) phosphorylated JNK (p-JNK) were analyzed by Western blot. Data are representative of three experiments.

Upon LPS stimulation, JNK is phosphorylated and activated by upstream MAPK/extracellular signal-regulated kinase kinase (MEK) [7,9,29]. The effect of EPA treatment on activated JNK levels was then determined. As expected, the amount of total JNKs was not altered by either LPS stimulation or EPA pretreatment (Fig. 4A). Lipopolysaccharide-induced JNK activation was decreased by preincubating cells with EPA (Fig. 4B).

4. Discussion

In the present study, we observed that pretreating THP-1 cells with EPA decreased LPS-stimulated DNA binding activity of AP-1. Inhibition of DNA binding activity of AP-1 by EPA supplementation was also reported in LPS-stimulated mouse macrophage cell line RAW 264.7 cells [30] and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated mouse epidermal cell line JB6 cells [31]. Our study further revealed that EPA prevented the LPS-stimulated increase of AP-1 components c-Jun and c-Fos. Therefore, the decrease of AP-1 DNA binding by EPA may partially attribute to the lowered amount of c-Jun and c-Fos.

The transcriptional activity of AP-1 results in part from the enhanced phosphorylation of c-Jun NH_2-terminal activation domain by JNK. Therefore, it is possible that EPA will alter AP-1 activity by modulating JNK activity. In contrast to the study of Liu et al. [31], in which EPA did not affect JNK activation in TPA-stimulated JB6 cells, our results showed that EPA decreased LPS-induced JNK activity and c-Jun phosphorylation in THP-1 cells. In Fig. 3A, the decrease of p-c-Jun by EPA appears much greater than the decrease of JNK activity (Fig. 3B). The explanation is that samples analyzed by Western blot (p-c-Jun) were normalized for equal amount of total proteins; therefore, the decrease of p-c-Jun is a combined effect of the lowered JNK activity and the decreased c-Jun level.

c-Jun N-terminal kinases, also called stress-activated protein kinases (SAPK), are a group of serine/threonine protein kinases that belong to the MAP kinase family. c-Jun N-terminal kinase can be activated by various stimuli such as environmental stress, proinflammatory cytokines and LPS [9,18,32]. In addition to increasing AP-1 activity through direct phosphorylation and activation of c-Jun [15,16,33], JNK can phosphorylate and activate Elk-1, resulting in an enhanced serum response element (SRE)-dependent c-*fos* expression [15,34]. TPA response elements in c-*jun* promoter are bound by activating transcription factor-2 (ATF-2) and c-Jun. c-Jun N-terminal kinase-mediated phosphorylation stimulates their transcriptional activities that lead to c-*jun* induction [15,35]. Thus, lowering JNK activity may be one of the mechanisms by which EPA decreases the up-regulation of c-Jun and c-Fos stimulated by LPS. Since newly synthesized c-Fos and c-Jun form stable AP-1 heterodimers that contribute to the augmentation of AP-1 activity upon stimuli, lowering JNK activity by EPA may also be responsible for its inhibition on DNA binding of AP-1.

Binding of c-Jun-containing complex is required for LPS induction of TNF-α promoter in THP-1 cells [12]. Therefore, modulation of JNK activity may affect TNF-α gene expression by affecting both the amount and the transcriptional activity of AP-1 components. c-Jun N-terminal kinase activity is also reported to be required for LPS-stimulated TNF-α translation [36]. By attenuating the LPS-induced JNK activity, EPA may modulate the transcription as well as the translation of TNF-α.

We previously observed a decreased DNA binding activity of NF-κB in LPS-stimulated THP-1 cells pretreated with EPA [28]. It is quite possible that EPA affects a common upstream pathway that leads to both NF-κB and AP-1 activation. In the serum, LPS first forms a complex with LPS-binding protein (LBP) and is presented to CD14 on the surface of myeloid cells [37]. The LPS–LBP–CD14 complex then interacts with toll-like receptor 4 (TLR) and its associated protein MD2, activating signal pathways that lead to the activation of MAPKs and IκB kinase [38–42]. It has been reported that LPS-stimulated CD14 expression and TLR-induced signaling pathways are down-regulated by n-3 fatty acids [43,44], providing mechanisms through which EPA may exert its effects on both AP-1 and NF-κB activation.

Both NF-κB and AP-1 are redox-sensitive transcription factors [45]. Cells stimulated with LPS have a 6- to 10-fold increase in releasing reactive oxygen species (ROS), including superoxide and hydrogen peroxide [46,47]. Previous findings in our laboratory demonstrated that supplementing fish oil to mice partially restored the tissue antioxidant defense systems that were suppressed by infection with murine leukemia virus [48]. Therefore, it is also possible that EPA may affect AP-1 and NF-κB activities via reducing ROS production induced by LPS.

Activator protein-1 elements have been found not only at TNF-α promoter, but also at a wide range of cytokine genes including interleukin-1, granulocyte-macrophage colony-stimulating factor and monocyte chemoattractant protein-1 [12,49–51]. Besides c-Jun, other JNK substrates include ATF-2, ternary complex factors Elk-1 and nuclear factor of activated T cell 4 (NFAT4) [52], and phosphorylation of these transcriptional factors may be modulated by EPA as well. Thus, by altering the DNA binding activity of AP-1, EPA may affect the expression of a variety of genes.

Our results suggest that EPA affects LPS-stimulated DNA binding activity of AP-1 at least through two ways: First, it may prevent the LPS-stimulated up-regulation of AP-1 components such as c-Jun and c-Fos. Second, it may decrease the LPS-induced phosphorylation of c-Jun. In conclusion, EPA may modulate LPS-stimulated TNF-α production partly through modulating AP-1 activation and JNK activity. Supplementation of fish oil or n-3 fatty acids such as EPA has been shown to alleviate the symptoms of rheumatoid arthritis and decrease the weight loss in patients with cancer and HIV infection [53–55]. The current study provides a possible molecular mechanism underlying the antiinflammatory effects of n-3 fatty acids and fish oil.

Acknowledgments

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