Docosahexaenoic acid inhibits inflammation via free fatty acid receptor FFA4, disruption of TAB2 interaction with TAK1/TAB1 and downregulation of ERK-dependent Egr-1 expression in EA.hy926 cells

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Scope: Inflammation is intimately associated with many cardiovascular events and docosahexaenoic acid (DHA) has been shown to protect against CVD. Egr-1 has emerged as a key regulator in the development of atherosclerosis. Free fatty acid receptor 4 (FFA4) is an n-3 FA membrane receptor. Tumor necrosis factor alpha (TNF-α) is an inflammatory mediator and transforming growth factor-β-activated kinase 1 (TAK1) is essential in the TNF-α-mediated activation of NF-κB. We examined the mechanisms underlying DHA inhibition of inflammation in human EA.hy926 cells.

Methods and results: TNF-α markedly induced the interaction between TAK1 binding protein (TAB) 2 and TAK1/TAB1, the phosphorylation of ERK, p38 MAPK and Akt, the expression of Egr-1 and ICAM-1, and HL-60 (monocyte-like) cell adhesion. Pretreatment with DHA attenuated TNF-α-induced phosphorylation of ERK, expression of Egr-1 and ICAM-1 and HL-60 cell adhesion. Transfection with siFFA4 reversed the DHA-mediated inhibition of TNF-α-induced Egr-1 and ICAM-1 expression, HL-60 cell adhesion and NF-κB and DNA-binding activity.

Conclusion: Our results suggest that the anti-inflammatory effect of DHA on the endothelium is at least partially linked to FFA4, disruption of TAB2 interaction with TAK1/TAB1 and downregulation of ERK-dependent Egr-1 and ICAM-1 expression, which leads to less HL-60 cell adhesion to TNF-α-stimulated EA.hy926 cells.

Keywords:
Docosahexaenoic acid (DHA) / Early growth response protein 1 (Egr-1) / Free fatty acid receptor 4 (FFA4) / Inflammation / Tumor necrosis factor alpha (TNF-α)

Introduction

Inflammation is well known for its role in many chronic diseases, including cardiovascular disease (CVD) [1], diabetes mellitus [2] and cancer [3]. CVD is the leading cause of death worldwide, accounting for 30% of all global deaths reported by the World Health Organization [4]. Leukocyte recruitment occurs in the early stage of vascular inflammation and plays a
pivotal role in the development of atherosclerosis. The adhesion and migration of monocytes to activated endothelial cells depends on the expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1) and selectins, which are upregulated by tumor necrosis factor alpha (TNF-α) [5,6]. TNF-α is an inflammatory cytokine that plays an important role in the development of atherosclerotic lesions. Plasma concentrations of TNF-α are elevated in several pathologies, including rheumatoid arthritis, cancer, atherosclerosis and pre-eclampsia [7]. TNF-α activates the IKK/NF-κB pathway, which regulates the expression of genes involved in inflammation, oxidative stress and endothelial dysfunction [8,9]. Disruption of the TNF-α gene diminishes the development of atherosclerosis in Apoe<sup>−/−</sup> mice via downregulation of ICAM-1, VCAM-1 and monocyte chemotactic protein 1 expression in the vascular wall, decreased expression of scavenger receptor type A and reduced oxLDL uptake in macrophages [10]. TNF-α also induces the expression of the transcription factors Ets-1, Egr-1 and c-fos, which participate in the formation of different forms of vascular lesions [11]. Egr-1 is among the transcription factors reported to induce the expression of ICAM-1, whose promoter has been shown to harbor functional Egr-1 DNA-binding motifs [12].

Egr-1 is an 80- to 82-kD inducible protein and belongs to the early growth response family of zinc finger proteins [13]. Ischemia/reperfusion, hyperoxia and hemorrhagic shock not only induce reactive oxygen species (ROS) mediated signaling and inflammation but also induce Egr-1 expression [14–16]. Transcriptional activation of Egr-1 and its downstream target genes is therefore critical in coordinating the cellular events that lead to inflammatory vascular damage [17]. Elevated expression of Egr-1 and Egr-1-inducible genes, such as IL-2, ICAM-1, and macrophage colony-stimulating factor 1, is noted in human atherosclerotic lesions compared to adjacent normal media [13]. Induction of Egr-1 expression by stressors is rapid and transient. Transcription of Egr-1 by heme is dependent on ERK1/2 activation, which leads to Elk-1 and NF-κB binding to the response elements in the Egr-1 promoter [17]. In an in vivo study, deletion of Egr-1 decreased the formation of atherosclerotic lesions in homozygous double-knockout mice (Egr<sup>1−/−</sup>/Apoe<sup>−/−</sup>) in the C57BL/6 background compared with mice deficient in Apoe alone [18].

NF-κB and AP-1, two critical inflammation- and redox-sensitive transcriptional factors, are activated by mitogen-activated protein kinase kinase kinases (MAP3Ks) [19]. Transforming growth factor-β-activated kinase 1 (TAK1) belongs to the MAP3K family [20] and is an important component of the TNF-α- and IL-1β-mediated activation of NF-κB [21]. In response to TNF-α and IL-1β, TAK1 activity is regulated by numerous regulatory subunits, including TAK1-binding protein 1 (TAB1), TAB2, TAB3 and TAB4 [22, 23]. The activated TAK1 induces the activation of IkB kinase, ERK, JNK and p38 MAPK and subsequently of the transcription factors NF-κB and AP-1. Activation of these transcription factors eventually upregulates the expression of many genes encoding proinflammatory cytokines, such as IL-6 [21]. Additionally, TAK1 plays a necessary role in TNFα-induced ERK activation, which controls the MAPK-EGFR signaling pathway and prevents cell apoptosis when facing the death signal in HeLa cells [24].

Docosahexaenoic acid (DHA) is found primarily in fish oil. Modest fish consumption has been associated with a reduced risk of CVD, and as such is considered to play a critical role in the prevention of CVD [25]. In previous studies, DHA was shown to have anti-inflammatory [26], antitumor [27] and immune-regulatory properties [28]. DHA not only suppresses various inflammatory markers [29], but also downregulates cytokine-induced expression of cellular adhesion molecules and even decreases monocyte adhesion to activated endothelial cells [26]. The anti-inflammatory effect of DHA has been well studied in our laboratory [26]. In addition to the induction of heme oxygenase 1 (HO-1) expression and suppression of NF-κB activation, we would like to unveil the other possible molecular mechanisms underlying the DHA-mediated inhibition of TNF-α-induced ICAM-1 expression in human vascular endothelial cells.

The G-protein-coupled receptors (GPCRs), which have seven transmembrane domains, constitute a large protein family of receptors that sense molecules outside the cell and activate intracellular signaling pathways and, ultimately, cellular responses, including stimulating insulin secretion and adipogenesis, regulating immune function, modulating glucose homeostasis, exerting anti-inflammatory activity and enhancing insulin sensitivity [30]. Expression of GPCRs is in a cell- or tissue-specific manner. For instance, FFA4 (GPCR120) is expressed in macrophages and mediates the potent anti-inflammatory and insulin-sensitizing effects of n-3 PUFAs [31].

Because of the roles of FFA4 in anti-inflammatory activity of n-3 PUFAs and Egr-1 in inflammatory vascular damage, the aim of this study was to test the roles of FFA4 and Egr-1 as well as involved mechanisms in the inhibition of TNF-α-induced ICAM-1 expression by DHA in endothelial cells.

2 Materials and methods

2.1 Reagents

Dulbecco’s modified Eagle medium (DMEM), RPMI-1640, phenol-red-free RPMI-1640, OPTI-MEM, penicillin/streptomycin and 25% trypsin-EDTA were from Gibco/BRL (Grand Island, NY, USA); fetal bovine serum (FBS) was from HyClone (Logan, UT); DHA was from Cayman Chemical (Ann Arbor, MI, USA); human TNF-α, Triton X-100, LY294002 and GW9508 were from Sigma-Aldrich (St. Louis, MO, USA); 4-[(4-Fluoro-4'-methyl[1,1'-biphenyl]-2-yl)methoxy]-benzenepropanoic acid (TUG-891), PD98059 and SB203580 were from TOCRIS (Ellisville, MO, USA); 2.7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM) was from CalBiochem (Billerica, MA, USA);...
TRIzol reagent was from Invitrogen (Carlsbad, CA, USA); antibodies against Egr-1, FFA4, JNK, phospho-JNK, ERK1/2, TAK1 and TAB1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and antibodies against ICAM-1, Akt, phospho-Akt (T308 and S473), phospho-ERK1/2, p38 MAPK, phospho-p38 MAPK and TAB2 were from Cell Signaling Technology (Boston, MA, USA).

2.2 Cell treatments

The human endothelial cell line EA.hy926 was a gift from Dr. T.S. Wang, Chung Shan Medical University, Taichung, Taiwan. Human leukemia promyelocytic cells (HL-60) were obtained from Bioresources Collection and Research Center (BCRC, Hsinchu, Taiwan). EA.hy926 cells were cultured in DMEM supplemented with 3.7 g/L NaHCO₃, 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. HL-60 cells were cultured in T-75 tissue culture flasks in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/L streptomycin. Cells were incubated at 37°C in a 5% CO₂ humidified incubator. EA.hy926 cells were allowed to grow to 80% confluence and were then treated with 100 μM DHA, which was prepared as described previously [26], for 16 h followed by treatment with 1 ng/mL TNF-α for various time periods.

2.3 Immunoprecipitation

Total cell lysates were diluted to 2 μg/μL with IP buffer (40 mM Tris-HCl, pH 7.5, 1% NP-40, 150 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 20 mM NaF, 1 μg/mL aprotinin, 1 μg/μL leupeptin and 1 mM sodium vanadate), incubated with 0.6 μg anti-TAK1 antibody for 16 h at 4°C, mixed with protein A-Sepharose (0.1 mg/mL), and incubated at 4°C for an additional 1 h. Immunoprecipitates were collected by centrifugation at 14 000 × g for 2 min. The pellet was washed with 200 μL of IP buffer three times and then subjected to Western blotting.

2.4 Western blotting analysis and electrophoretic mobility shift assay (EMSA)

After each experiment, cells were washed twice with cold PBS before lysis and were harvested in 150 μL of lysis buffer (10 mM Tris-HCl, pH 8, 0.1% Triton X-100, 320 mM sucrose, 5 mM EDTA, 1 mM PMSF, 1 mg/L leupeptin, 1 mg/L aprotinin and 2 mM dithiothreitol). Cell homogenates were centrifuged, and the resulting supernatant was used as a cellular protein for Western blotting analysis. Nuclear protein preparation was performed as described previously [32]. The total protein was analyzed by use of the CoomassiePlus protein assay reagent kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of cellular proteins were applied to each well in a sodium dodecyl sulfate-10% polyacrylamide gel. After electrophoresis, the separated proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Nonspecific binding sites on the membranes were blocked with 5% nonfat dry milk in 15 mM Tris/150 mM NaCl buffer (pH 7.4) at room temperature for 2 h. After blocking, the membranes were incubated with antibodies overnight. Thereafter, the membranes were incubated with the secondary peroxidase-conjugated anti-rabbit IgG at 37°C for 1 h. The bands were visualized by using an enhanced chemiluminescence kit (PerkinElmer Life Science, Boston, MA, USA) and were scanned with a luminescent image analyzer (LAS-4000, FUJIFILM, Japan). The bands were quantitated with ImageGauge software (FUJIFILM). EMSA was performed according to our previous study [32].

2.5 RNA isolation and RT-PCR

Total RNA was isolated from EA.hy926 cells by using TRIzol reagent. After treatment, cells were washed twice with cold PBS and scraped with 500 μL of TRIzol reagent. Cell samples were mixed with 100 μL of chloroform and were allowed to react at room temperature for 5 min and were then centrifuged at 11 000 × g for 15 min. After centrifugation, the supernatant was discarded and the cell pellet was stored in 70% ethanol or dissolved in deionized water for quantification. Amounts of 0.2 μg total RNA were used for the synthesis of first-strand cDNA by using Moloney murine leukemia virus reverse transcriptase (Promega) in a final volume of 20 μL containing 5 mM MgCl₂, 1 mM of each deoxynucleotide triphosphate, 2.5 mM oligo(dT) and 40 U of RNase inhibitor. PCR amplification was conducted in a thermocycler in a reaction volume of 50 μL containing 20 μL of cDNA, BioTaq PCR buffer, 4 mM MgCl₂, 1 U of BioTaq DNA polymerase (BioLine) and 6 pmol forward and reverse primers. Oligonucleotide primers were as follows: ICAM-1 (forward, 5′-TGAAGGCCACCCCAAGAGCCACAC-3′; reverse, 5′-CCCATTGACTCGGCTGCTGCTACC-3′), Egr-1 (forward, 5′-TGCACCCTCTCAGTTGTTCC-3′; reverse, 5′-AAAGACTCTGGTCAGGGT-3′), and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (forward, 5′-CACTCACATCTTCCAGGAG-3′; reverse, 5′-CTGTTCACCACCTTCTTG-3′). Amplification of ICAM-1 was achieved when samples were heated to 95°C for 5 min and then immediately cycled 32 times through a 1-min denaturing step at 94°C, a 1-min annealing step at 56°C and a 1-min elongation step at 72°C. Amplification of Egr-1 was achieved when samples were heated to 95°C for 5 min and then immediately cycled 40 times through a 1-min denaturing step at 95°C, a 1-min annealing step at 51°C and a 1.5-min elongation step at 72°C. The GAPDH cDNA level was used as the internal standard.

2.6 HL-60 cell adhesion assay

EA.hy926 cells in six-well plates were allowed to grow to 80% confluence and were then pretreated with 100 μM DHA for
16 h followed by incubation with 1 ng/mL TNF-α for an additional 6 h. Afterward, the HL-60 cell adhesion assay was performed according to our previous study [33].

2.7 Reactive oxygen species measurement

Detection of intracellular ROS was performed by using the probe 2,7-dichlorofluorescin diacetate (H$_2$DCFDA; Invitrogen, Grand Island, NY, USA) as described previously [34]. Briefly, cells were grown to 60–70% confluence and were then serum-starved in DMEM supplemented with 0.5% (v/v) FBS for an additional 1 day. After this, cells were pretreated with 100 μM DHA for 16 h, followed by incubated for 10 min with the ROS-sensitive fluorophore H$_2$DCFDA (10 μM), and finally challenged with TNF-α for 20 min. Cells were immediately observed under a laser-scanning confocal microscope (Leica TCS SP2). DCF fluorescence was excited at 488 nm with an argon laser, and the evoked emission was filtered with a 515 nm long pass filter.

2.8 RNA interference by small hairpin RNA of Egr-1

Lentiviral infection was performed according to the method of a previous study [35]. Two different sequences targeting human Egr-1 mRNA were chosen and purchased from the National RNAi Core facility platform (Taipei, Taiwan). RNAi clones were identified by their unique number assigned by the RNAi Consortium (TRCN) as follows: TRCN0000013834 (responding sequence: CGACATCTGTGGAGAAAGTT) was used for shEgr-1 (1) targeted to Egr-1, TRCN0000013836 (responding sequence: CATCTCTGAAACAAGGAAA) was used for shEgr-1 (2) targeted to Egr-1, and the TRCN00000772246 (responding sequence: CAAATCACAGAATCGTCGTAT) was used for vector control targeted to luciferase. Briefly, cells were plated onto 6-cm plastic culture dishes in DMEM supplemented with 1.5 g/L sodium bicarbonate, 100 IU penicillin/mL, 100 μg/mL streptomycin and 10% FBS. After a 24-h attachment period, the cells were infected with packaged lentviruses for another 24 h. On the following day, the medium was removed, and the cells were selected by using 2 μg/mL puromycin for 2 days. The cells were then passaged to 10-cm plastic culture dishes and were ready for assay.

2.9 RNA interference by small interfering RNA (siRNA) of FFA4

Predesigned siRNAs against human FFA4 and nontargeting control-pool siRNA were purchased from Dharmacon Inc. (Lafayette, CO, USA). The four siRNAs against the human FFA4 gene were as follows: (a) GAAAUGACUUGUCCGUUAU, (b) CAAGACGUGUCCGACUA, (c) GGACUGGUCAUUGUCAU and (d) GGAAGAGGCUACCGGUAAG. Cells were transfected with FFA4 siRNA SMARTpool by using DharmaFECT1 transfection reagent (Thermo) according to the manufacturer’s instructions. Nontargeting siRNA construct (NTC) was used as a negative control. Specific silencing was confirmed by at least three independent Western blotting assays with cellular extracts 16 h after transfection.

2.10 Plasmids, transfection and luciferase assay

The ICAM-1 promoter-luciferase construct (pIC339, -339 to 0) was a gift from Dr. P.T. van der Saag (Hubrecht Laboratory, Utrecht, the Netherlands). pIC339 contains NF-κB (−187/−178), AP-1 (−284/−279), AP-2 (−48/−41) and Sp1 (−59/−53, −206/−201) binding sites [36]. All subsequent experiments were performed according to our previous study [26].

2.11 Statistical analysis

Data were analyzed by using analysis of variance (SAS Institute, Cary, NC, USA). The significance of the difference in mean values was determined by one-way analysis of variance followed by Tukey’s test or by a two-tailed Student’s t-test. $p$ Values < 0.05 were taken to be statistically significant.

3 Results

3.1 Effect of TNF-α on Egr-1 gene expression

To determine whether TNF-α induces Egr-1 gene expression, we treated EA.hy926 cells with 1 ng/mL TNF-α for various time periods. TNF-α induced Egr-1 protein expression and peak induction was observed at 60 min (Fig. 1A). TNF-α induced peak Egr-1 mRNA expression at 30 min (Fig. 1B). Thus, the induction of Egr-1 by TNF-α was rapid and transient.

3.2 TAB2 binding to TAK1/TAB1 and ERK activation are involved in TNF-α-induced expression of Egr-1 and ICAM-1 as well as HL-60 cell adhesion

TNF-α is a well-known inflammatory mediator. We then investigate the mechanisms by which TNF-α induces inflammation in EA.hy926 cells. In resting conditions, TAK1, a member of the MAP3K family, is endogenously co-immunoprecipitated with TAB1, which suggests that the molecular interaction might lead to maintenance of the preferential inactive form of TAK1. However, the interaction between TAK1 and TAB2 is significantly increased upon TNF-α treatment and TAK1 is activated [19]. In this study, we found that the interaction between TAK1 and TAB2 was increased at 10 min in the presence of TNF-α, whereas pretreatment with DHA for 16 h attenuated the interaction (Fig. 2A).
result suggested that attenuation of TNF-α-induced interaction between TAK1 and TAB2 was involved in the inhibition of TNF-α-induced inflammation by DHA.

To investigate the signaling pathways involved in the TNF-α-induced expression of Egr-1 and ICAM-1, we examined the effect of TNF-α on phosphorylation of the MAPKs and PI3K/Akt. Phosphorylation of Akt, ERK and p38 MAPK was observed, although their phosphorylation peak varied (Fig. 2B). To further delineate the roles of PI3K/Akt, ERK and p38 MAPK, we pretreated cells with LY294002, PD98059 and SB203580, respective inhibitors of PI3K/Akt, ERK and p38 MAPK, for 1 h. We then challenged the cells with 1 ng/mL TNF-α for another 1 h for the Egr-1 expression assay and for another 6 h for the ICAM-1 expression and HL-60 cell adhesion assays. As shown in Figs. 2C and D, inhibitors of ERK and PI3K/Akt abolished TNF-α-induced Egr-1 protein and mRNA expression. ICAM-1 protein and mRNA expression, by contrast, was attenuated only by ERK inhibitor. Although SB203580 had no effect on TNF-α-induced expression of Egr-1 and ICAM-1, it decreased the adhesion of HL-60 cells to TNF-α-stimulated EA.hy926 cells similar to the activity of PD98059 (Fig. 2E). These results suggested that the TNF-α induction of ICAM-1 expression and HL-60 cell adhesion was mediated by the ERK/Egr-1/ICAM-1 cascade. The inhibition of HL-60 cell adhesion to TNF-α-stimulated EA.hy926 cells by SB203580 was Egr-1/ICAM-1-independent, however.

3.3 Silencing of Egr-1 attenuates TNF-α-induced ICAM-1 expression and HL-60 cell adhesion

Increasing evidence supports that Egr-1 and Egr-1-inducible genes are expressed at high levels in mouse and human atherosclerosis [13]. We hypothesized that the induction of ICAM-1 expression by TNF-α might be associated with the induction of Egr-1. To study this, we used an shRNA system to knock down Egr-1. Compared with cells transfected with shLuc, cells transfected with shEgr-1 showed a reduction in Egr-1 protein and mRNA expression upon TNF-α exposure (Fig. 3A). Furthermore, TNF-α-induced ICAM-1 protein and mRNA expression as well as HL-60 cell adhesion to TNF-α-stimulated EA.hy926 cells were significantly inhibited in cells transfected with shEgr-1 (Figs. 3B–D). Thus, HL-60 cell adhesion to TNF-α-stimulated endothelial cells is associated with Egr-1 expression, which leads to the increased expression of ICAM-1. Our results suggest that Egr-1 plays a pivotal role in vascular inflammation.

3.4 DHA inhibits TNF-α-induced ROS generation, ERK phosphorylation and Egr-1 expression

Our previous study showed that DHA inhibited TNF-α-induced ICAM-1 expression was through the induction of HO-1 [26]. In this study, we focused on the role of Egr-1 in the inhibition of TNF-α-induced ICAM-1 expression by DHA. As shown in Fig. 4A, DHA pretreatment for 16 h inhibited TNF-α-induced cellular ROS generation which is possibly implicated in cellular signaling transduction. Thereafter, cells were pretreated with 100 μM DHA for 16 h before being challenged with 1 ng/mL TNF-α for an additional 30 min for ERK phosphorylation measurement. As shown in Fig. 4B, DHA pretreatment inhibited TNF-α-induced ERK phosphorylation. In addition, we pretreated cells with 100 μM DHA for 16 h and then challenged cells with 1 ng/mL TNF-α for an additional 60 min for Egr-1 protein measurement. As shown, DHA pretreatment inhibited TNF-α-induced Egr-1 protein (Fig 4C) and mRNA (Fig. 4D) expression. Moreover, DHA pretreatment for 16 h inhibited TNF-α-induced Egr-1 mRNA expression (Fig. 4D). These results suggest that DHA inhibits TNF-α-induced ERK-dependent Egr-1 gene expression.

3.5. Inhibition of TNF-α-induced Egr-1 and ICAM-1 expression as well as HL-60 cell adhesion by DHA is mediated by FFA4

DHA has been shown to have potent anti-inflammatory activity, and the underlying mechanisms involved are studied extensively. However, there are few studies indicating...
Figure 2. TAB2 binding to TAK1/TAB1 and ERK activation are involved in TNF-α-induced expression of Egr-1 and ICAM-1 and HL-60 cell adhesion. Cells were pretreated with 100 μM DHA for 16 h before being challenged with 1 ng/mL TNF-α for 10 min. (A) Cells were extracted and immunoprecipitated with anti-TAK1 antibody. The interaction was detected by Western blotting with anti-TAB1 and anti-TAB2 antibodies. The same lysates were verified with anti-TAK1 antibody. (B) Cells were incubated with 1 ng/mL TNF-α for the indicated time periods. Immunoblots of total protein extracted from treated cells were then probed with antibodies specific to Akt and the MAPKs. (C) Cells were pretreated with 20 μM PD (PD98059), SB (SB203580), or LY (LY294002) for 1 h before being challenged with 1 ng/mL TNF-α for another 1 h for Egr-1 protein measurement and for another 6 h for ICAM-1 protein measurement. Aliquots of total protein (10 μg) were used for Western blot analysis. (D) Total RNA was isolated from treated cells and was subjected to RT-PCR. (E) Cells were pretreated with kinase inhibitors for 1 h before being challenged with 1 ng/mL TNF-α for another 6 h, and were assessed for HL-60 cell adhesion. Values are means ± SD of three independent experiments. Values not sharing the same letter are significantly different (p < 0.05).

the possible receptors mediating the anti-inflammatory activity of DHA. FFA4, a membrane receptor of n-3 FAs in macrophages and adipocytes, contributes to the potent anti-inflammatory effect of n-3 FAs [31]. FFA4 was expressed in EA.hy926 cells and in the positive controls THP-1 (a human acute monocytic leukemia cell line) [37] and MCF-7 (a human breast cancer cell line) [38] (Fig. 5A). A dose-response study comparing the effects of two synthetic FFAR agonists, the FFA1-selective agonist, GW9508 and the FFA4-selective agonist, TUG-891, on TNF-α-induced ICAM-1 expression was performed. As shown in Fig. 5B, both GW9508 and TUG-891 dose-dependently inhibited TNF-α-induced ICAM-1 expression. The relatively low potency of GW9508 was consistent with its previously reported ability to activate FFA4 at micromolar doses, while the higher potency of TUG-891 was consistent with its action at FFA4 [39]. GW9508 was used in subsequent experiments to test whether the inhibitory effect of DHA on TNF-α-induced Egr-1 and ICAM-1 expression was via an FFAR. Cells were pretreated with 100 μM DHA for 16 h or 1 μM GW9508 for 1 h before being challenged with 1 ng/mL TNF-α for an additional 1 h for Egr-1 protein expression. As shown in Fig. 5C, pretreatment with GW9508 or DHA inhibited TNF-α-induced Egr-1 protein expression. Moreover, cells were pretreated with 100 μM DHA for 16 h or 1 μM GW9508 for 1 h before being challenged with 1 ng/mL TNF-α for an additional 6 h for ICAM-1 gene
expression measurements. As shown in Figs. 5D and E, pretreatment with GW9508 or DHA inhibited TNF-α-induced ICAM-1 protein and mRNA expression. Considering GW9508 is a selective agonist for FFA1, we additionally used TUG-891 to verify the role of FFA4 in DHA inhibition of inflammation in this study. Hudson and colleagues [39] demonstrate that TUG-891 is highly selective for FFA4 in human cells. As shown in Fig. 5F, pretreatment with TUG-891 inhibited TNF-α-induced ICAM-1 protein expression as DHA did. As shown in Fig. 5G, DHA and GW9508 inhibited HL-60 cell adhesion to TNF-α-stimulated EA.hy926 cells. In addition, we performed a promoter activity assay by using a human ICAM-1 promoter-luciferase construct, pIC339 (-339 to 0). TNF-α-induced ICAM-1 promoter activity was inhibited by DHA and GW9508 (Fig. 5H). TNF-α-induced nuclear translocation of p65 is critical for ICAM-1 gene transcription, and this effect was also inhibited by DHA and GW9508 (Fig. 5I). The pretreatment period for DHA and GW9508 varies in different studies. As shown by Yan and colleagues [37], the inhibitory effects of DHA and GW9508 on nigericin-induced inflammation are after 3-h incubation.

Moreover, the role of FFA4 in the inhibition of TNF-α-induced Egr-1 and ICAM-1 expression by DHA was clarified by using the siRNA system to create a FFA4 knockdown model. The efficiency of the siRNA system to silence FFA4 was ascertained by Western blot (Fig. 6A). As shown in Figs. 6B–F, FFA4 siRNA attenuated the inhibition of TNF-α-induced Egr-1 protein and ICAM-1 gene expression, HL-60 cell adhesion and p65 nuclear translocation by DHA. These findings implicate the involvement of FFA4 in the inhibition of TNF-α-induced inflammation by DHA in endothelial cells.

4 Discussion

Inflammation is an etiology of many chronic diseases [1–3], and TNF-α is well known for its pro-inflammatory properties. DHA, a major component of fish oil, is implicated in anti-inflammation [26], immunomodulation [40] and anti-carcinogenesis [27]. Our laboratory showed previously that the anti-inflammatory activity of DHA is associated with HO-1 induction and IKK/NFκB inhibition [26]. In the present...
study, we demonstrated that the anti-inflammatory effect of DHA was dependent on FFA4, disruption of TAB2 interaction with TAK1/TAB1, and down-regulation of ERK-dependent Egr-1 and ICAM-1 expression, as well as HL-60 cell adhesion to TNFα activated endothelial cells.

Egr-1, a major vascular pathogenic transcription factor in atherosclerosis [13, 41], is induced by cytokines, hypoxia, physical forces and injurious stimuli [13]. The role of Egr-1 in atherosclerosis has been demonstrated in an animal study. Harja et al. [18] found that deletion of Egr-1 decreased atherosclerotic lesions in homozygous double-knockout mice (Egr1−/−/Apoe−/−) in the C57BL/6 background compared with mice deficient in ApoE alone. One of the genes induced by Egr-1 is ICAM-1 [12]. Because Egr-1 is an important mediator of TNFα-induced inflammation, in the present study, we examined the effect of TNFα on Egr-1 expression. As shown in Fig. 1, TNFα induced Egr-1 expression in a rapid and transient manner. Moreover, we found that TNFα-induced ICAM-1 expression and HL-60 cell adhesion were mediated by Egr-1 (Fig. 3B–D). Knockdown of Egr-1 by shEgr-1 attenuated TNFα-induced ICAM-1 expression and HL-60 cell adhesion.

The activation of numerous signaling pathways, including the ERK1/2, JNK, p38 MAPK and PI3K/Akt pathways, is implicated in the pro-inflammatory action of TNFα. For example, TNFα is a strong regulator of matrix metalloproteinase (MMP) and tissue inhibitor (TIMP) expression in trabecular meshwork [42]. Inhibition of ERK phosphorylation blocks this TNFα-induced MMP and TIMP expression, and manipulation of this signal transduction pathway may
Figure 5. FFA4 mediates DHA inhibition of TNF-α-induced Egr-1 and ICAM-1 expression, HL-60 cell adhesion and NF-κB and DNA-binding activity. (A) FFA4 is expressed in THP-1, MCF-7 and EA.hy926 cells. THP-1 and MCF-7 cells were used as the positive control. (B) Both GW9508 and TUG-891 dose-dependently inhibited TNF-α-induced ICAM-1 protein expression. Cells were pretreated with GW9508 (1–20 μM) or TUG-891 (0.1–10 μM) for 1 h and were then incubated with 1 ng/mL TNF-α for another 6 h. Aliquots of total protein (10 μg) were used for ICAM-1 protein measurement. Cells were pretreated with 100 μM DHA for 16 h or 1 μM GW9508 for 1 h and were then incubated with 1 ng/mL TNF-α for another 1 h or 6 h. Aliquots of total protein (10 μg) were used for Western blot analysis, and expression of Egr-1 (C) and ICAM-1 (D) was assessed. (E) Total RNA was analyzed by RT-PCR. (F) Cells were pretreated with 100 μM DHA for 16 h or 10 μM TUG-891 for 1 h and were then incubated with 1 ng/mL TNF-α for another 6 h. Aliquots of total protein (20 μg) were used for Western blot analysis. (G) Cells were pretreated with 100 μM DHA for 16 h or 1 μM GW9508 for 1 h followed by incubation with 1 ng/mL TNF-α for another 6 h and were assessed for HL-60 cell adhesion. (H) Cells transfected with the pIC339 luciferase expression vector were pretreated with 100 μM DHA for 16 h or 1 μM GW9508 for 1 h followed by incubation with 1 ng/mL TNF-α for another 6 h. Cells were then lysed and analyzed. Luciferase activity was assayed as described in Materials and methods. Induction is shown as an increase in the normalized luciferase activity in the treated cells relative to the control. (I) Cells were pretreated with 100 μM DHA for 16 h or 1 μM GW9508 for 1 h followed by incubation with 1 ng/mL TNF-α for another 3 h. Aliquots of nuclear extracts (10 μg) were used for EMSA. Values are means ± SD of three independent experiments. Values not sharing the same letter are significantly different (p < 0.05).

provide a target for developing improved glaucoma treatments. Also, TNFα-induced MMP-9 expression is mediated by p38 MAPK in human urinary bladder cancer 5637 cells and this induction is inhibited by the p38 MAPK inhibitor SB203580 [43]. It has been demonstrated that TNFα-induced Lnk expression is through the PI3K/Akt pathway in HUVECs and that the PI3K inhibitors LY294002 and Wortmannin abolish the induction effect of TNF-α [44].

TNF-α increases the expression of adhesion molecules such as E-selectin, VCAM-1 and ICAM-1, which are responsible for the adhesion and migration of monocytes to activated endothelial cells [5, 6]. In the present study, TNF-α activated the PI3K/Akt, ERK, and p38 MAPK signaling pathways (Fig. 2B). To determine the contribution of respective signaling pathways to TNF-α-induced Egr-1 and ICAM-1 expression and HL-60 cell adhesion, respective inhibitors of
P13K/Akt, ERK, and p38 MAPK were used. As shown in Fig. 2–E, PD98059 inhibited TNFα-induced Egr-1 and ICAM-1 expression as well as HL-60 cell adhesion. LY294002, however, inhibited TNF-α-induced Egr-1 expression other than ICAM-1 expression and HL-60 cell adhesion. These results suggest that ERK-dependent Egr-1 expression is involved in TNF-α-induced ICAM-1 expression and HL-60 cell adhesion. Unexpectedly, the p38 MAPK inhibitor SB203580 inhibited TNFα-induced HL-60 cell adhesion. This implies that the role of p38 MAPK in TNFα-induced HL-60 cell adhesion is likely Egr-1/ICAM-1-independent. It was reported that the TNF-α-induced Egr-1 protein expression depends on the ERK pathway but not on the p38 MAPK pathway in Hela cells [45]. Our result was consistent with that of Shin and colleagues [45]. However, the p38 MAPK pathway was found to be involved in the TNF-α-induced E-selectin expression and leukocyte diapedesis [46]. This might explain why the p38 MAPK pathway had no effect on TNF-α-induced Egr-1 and ICAM-1 expression but played a role in the TNF-α-induced HL-60 cell adhesion. Because ICAM-1 is not the only adhesion molecule involved in monocyte adhesion. Egr-1 is among the transcription factors for ICAM-1 expression. It suggests that the inhibition of Egr-1 expression does not definitely lead to reduced ICAM-1 expression. Our data showed that LY294002 significantly inhibited the TNF-α-induced Egr-1 expression but not ICAM-1 expression and HL-60 cell adhesion (Fig. 2C–E). Madge and Pober [47] showed that TNF-α significantly increased ICAM-1 promoter–reporter gene activity in human umbilical vein endothelial cells; however, pretreatment with LY294002 did not show any significant difference.
Figure 7. Scheme summarizing the inhibition of TNF-α-induced vascular inflammation by DHA via FFA4, disruption of TAB2 interaction with TAK1/TAB1, and downregulation of ERK-dependent NF-κB activation and Egr-1 and ICAM-1 expression, which lead to attenuation of HL-60 cell adhesion to TNF-α-stimulated EA.hy926 cells.

in ICAM-1 promoter-reporter gene activity compared with untreated cells. Also, LY294002 did not attenuate TNF-α-induced NF-κB p65 subunit binding activity in HAECs [48]. NF-κB is an important transcription factor for ICAM-1 transcription [49].

GPCRs (40, 41, 43, 84, 119 and 120) are membrane proteins characterized by a common motif of seven transmembrane domains [50]. Ligand binding to receptors leads to receptor conformational changes and activation of intracellular G-proteins. Different types of G-protein coupling determine the activation of distinct downstream signaling pathways [50]. In recent years, the roles of GPCRs in the action of fatty acids in different tissues have been extensively studied. FFA4, a physiological receptor of n-3 FAs, is highly expressed in macrophages and adipocytes; however, no evidence has demonstrated the presence of FFA4 in EA.hy926 cells. To show that FFA4 is involved in the anti-inflammatory activity of DHA in endothelial cells, we determined the protein level of FFA4 in EA.hy926 cells (Fig. 5A). Moreover, we showed that transfection with FFA4 siRNA knocked down the FFA4 protein expression in EA.hy926 cells (Fig. 6A). FFA4 has been shown to mediate the potent anti-inflammatory effects of EPA and DHA in macrophages [31], and the underlying mechanism by which activated FFA4 inhibits inflammation in macrophages was proposed by Talukdar et al. [30]. TNF-α is a pro-inflammatory agent, and it induces inflammation by binding to TNF-α receptor 1 (TNFR1). Upon binding of TNF-α, TNFR1 recruits several adaptors, including TRAF2, cIAP1/2 and RIPK1, to form a complex that results in Lys63-linked polyubiquitination of TRAF2 and RIPK1. It is reported that the Lys63-linked TRAF2 polyubiquitination further recruits and activates TAK1 through binding of the TAB2 and TAB3 to the Lys63-polyubiquitinated chains [51]. The interaction between TAB2/TAB3 and TAK1 is critical for the activation of signaling cascades mediated by TNF-α [52]. In the present study, DHA blocked the interaction between TAB2 and TAK1/TAB1 induced by TNF-α (Fig. 2A). This result suggests that...
inhibition of the association of TAB2 with the TAK1/TAB1 complex at least partially explains the anti-inflammatory activity of DHA. DHA pretreatment inhibited TNF-α-induced Egr-1 and ICAM-1 expression, ICAM-1 promoter activity and HL-60 cell adhesion (Fig. 5C–H), and siFFA4 attenuated the inhibition of TNF-α-induced Egr-1 and ICAM-1 expression as well as HL-60 cell adhesion by DHA (Fig. 6B–E). Moreover, GW9508, an FFAR agonist, showed a similar effect on TNF-α-induced Egr-1 and ICAM-1 expression, ICAM-1 promoter activity, and HL-60 cell adhesion to DHA (Fig. 5C, D, E, G and H). TUG-891, a potent and selective agonist of the FFA4, suppressed TNF-α-induced ICAM-1 protein expression similar to the activity of DHA (Fig. 5F). These results suggest that the inhibition of TNF-α-induced vascular inflammation by DHA is likely associated with FFA4.

DHA is well-known for its cardioprotective effects. However, the mechanisms of DHA’s protection against CVD are not fully understood. A couple of mechanisms have been proposed, including preventing arrhythmias, lowering plasma triacylglycerols [53], reducing blood pressure [54], decreasing platelet aggregation [55], improving vascular reactivity [56] and reducing inflammation [57]. Regarding the mechanisms involved in the anti-inflammatory effect of n-3 FAs include alteration of cell membrane fatty acid composition [58], reduction of expression of adhesion molecules such as ICAM-1 and E-selectin [59], downregulation of LPS-induced NF-κB activation via activation of the PPAR-γ pathway [60], and induction of the NrF2/HO-1 pathway leading to inhibition of NF-κB activation [26]. In this study, we provide new insight into the anti-inflammatory effect of DHA on endothelial cells. We showed that DHA exerted its effect by suppressing TNF-α-induced ERK-dependent Egr-1 and ICAM-1 expression, as well as HL-60 cell adhesion via FFA4.

The findings of this study are presented schematically in Fig. 7. FFA4 is involved in the inhibition of TNF-α-induced inflammation by DHA in endothelial cells. The working mechanisms of DHA comprise disruption of TAB2 interaction with TAK1/TAB1, downregulation of ERK-dependent NF-κB activation and Egr-1 and ICAM-1 expression and attenuation of HL-60 cell adhesion. It has been shown that the ERK plays a critical role in TNF-α-induced NF-κB activation [61], which interacts with the Egr-1 promoter and upregulates Egr-1 expression [17]. The anti-inflammatory activity of DHA in vascular endothelium partially contributes to the anti-atherosclerotic effect of DHA.

H. W. C. and C. K. L. designed the study; K. L. L., Y. C. Y., H. T. Y., T. W. C., C. Y. L., C. C. L. and H. J. T. conducted the research; and C. K. L. analyzed the data. C. K. L. and H. W. C. wrote the manuscript. C. K. L. and H. W. C. have the primary responsibility for the final content. All of the authors have read and approved the final manuscript.

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5 References


