Research Article

Docosahexaenoic acid reduces linoleic acid induced monocyte chemoattractant protein-1 expression via PPARγ and nuclear factor-κB pathway in retinal pigment epithelial cells

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Scope: To investigate whether docosahexaenoic acid (DHA) could inhibit linoleic acid (LA) induced monocyte chemoattractant protein (MCP)-1 expression in human retinal pigment epithelial (RPE) cells.

Methods and results: ARPE-19 cells were pretreated with DHA and then exposed to LA. The expression of MCP-1 and PPARγ was examined using RT-PCR and Western blot analysis. LA at 10, 25, or 50 μM induced expression of MCP ARPE-19 cells in a dose-dependent manner (p < 0.05). DHA at 50 and 100 μM effectively inhibited LA-induced MCP-1 expression and production (p < 0.05) and NF-κB activation. In addition, the culture medium from LA-stimulated ARPE-19 cells could induce tube formation in choroidal endothelial cells (RF6A), whereas 100 μM DHA inhibited tube formation. DHA at 100 μM increased the expression and activity of PPARγ (p < 0.05). Pretreatment with PPARγ inhibitor (GW9662) abolished the inhibitory effect of DHA (100 μM) on LA-induced IκB degradation, p65 translocation, and MCP-1 expression in ARPE-19 cells (p < 0.05), as well as tube formation in RF6A.

Conclusion: DHA reduced LA-induced MCP-1 expression via a PPARγ- and NF-κB-dependent pathway in ARPE-19 cells. These results suggest the molecular mechanisms underlying the beneficial effects of increased consumption of DHA and reduced consumption of LA on age-related macular degeneration.

Keywords:
Docosahexaenoic acid / Linoleic acid / Monocyte chemoattractant protein-1 / Neovascularization / Peroxisome proliferator-activated receptors

1 Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in patients over 50 years of age [1].

The majority of these patients who have severe vision loss have macular damage caused by choroidal neovascularization (CNV), which is defined as the abnormal growth of new choroidal vessels under retinal pigment epithelial (RPE) cells and the retina. Dietary fatty acid consumption is widely accepted as a behaviorally modifiable risk factor for AMD [2, 3]; thus, modification of dietary fatty acid intake could be a promising approach to prevent the development of AMD [4, 5].

PUFAs can be divided into two major groups: ω-3 and ω-6 PUFAs [6]. Linoleic acid (LA) and arachidonic acid (AA) are the major ω-6 PUFAs, and docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the major ω-3 PUFAs. Several epidemiologic studies of fatty acid intake have found that higher levels of ω-3 fatty acids are associated with a reduced risk for AMD, but only when levels of LA (an ω-6 fatty acid) are low [7, 8]. Some explanations have been proposed, including...
the competition of desaturation enzymes between ω-6 and ω-3 fatty acids, with high intake of LA thereby resulting in the production of more proinflammatory eicosanoids that contribute to the pathogenesis of AMD. However, it is generally accepted that the impairment of RPE cell function is a crucial step in the molecular pathways leading to clinically relevant AMD. The cardinal function of RPE cells is the phagocytic uptake and degradation of constantly shed apical photoreceptor outer segments (POS) [9]. Because POS are rich in PUFAs, the phagocytic function directly exposes RPE cells to various types of fatty acids. Furthermore, several reports showed that dietary fatty acid intake influences the fatty acid profiles in the retina [10, 11], indicating that dietary fatty acids may influence the concentrations of fatty acid exposed to RPE cells. Therefore, understanding the effects of ω-3 and ω-6 PUFAs on RPE cells and their possible regulatory mechanisms may help to clarify the roles of dietary ω-3 and ω-6 PUFAs in the pathogenesis of AMD.

Increasing evidence has shown that inflammation plays a central role in the development of AMD [12, 13]. Inflammatory mediators, such as monocyte chemoattractant protein-1 (MCP-1) and vascular endothelial growth factor released from RPE cells are involved in CNV formation [14,15]. Studies have demonstrated that the intraocular concentration of inflammatory cytokines such as MCP-1 is significantly associated with CNV, even in the presence of normal vascular endothelial growth factor concentrations [16]. In addition, MCP-1 knockout mice demonstrated a reduction in the number of infiltrating macrophages and an amelioration of CNV formation [17].

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of a family of nuclear receptors [18]. PPARγ is activated by both natural ligands, such as PUFAs, and by synthetic ligands, such as thiazolidinediones [19,20]. PPARγ activation is anti-inflammatory in nature and has been shown to play a protective role in various tissues [21,22]. Increasing evidence has demonstrated that the mechanism of the anti-inflammatory action of PPARγ ligands has partly been attributed to inhibition of the NF-κB pathway [23].

In this study, we investigated the effects of the ω-3 PUFAs DHA and EPA and the ω-6 PUFAs LA and AA on the expression of MCP-1 in ARPE-19 cells. We hypothesized that LA could induce CNV through the activation of NF-κB and MCP-1 expression. DHA could reduce LA-induced MCP-1 expression by activating PPARγ and then inhibiting the NF-κB signaling pathway.

2 Materials and methods

2.1 Reagents

LA, EPA, DHA, AA, GW9662, and troglitazone were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-MCP-1 antibody was obtained from Calbiochem (San Diego, CA, USA). Anti-NF-κB p65 antibody was purchased from Santa Cruz (Dallas, TX, USA).

2.2 Cell culture

ARPE-19 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Sigma-Aldrich) with 10% fetal calf serum in a humidified incubator at 37°C in an atmosphere of 5% CO₂. Cells were used at the third to fifth passages.

2.3 Fatty acid preparation and treatment

Fatty acids were dissolved in ethanol, and the final ethanol concentration in the medium was at most 0.5% v/v. The fatty acids were added to the culture medium together with 10% fetal calf serum and incubated in a shaking water bath at 37°C for 1 h. The fatty acid containing medium was adjusted to the final volume with DMEM culture medium and used immediately. Cells were stimulated with vehicle or with 10, 25, or 50 μM LA or AA; or with 25, 50, or 100 μM DHA or EPA for 12 h. To investigate the inhibitory effects of DHA, cells were incubated for 12 h with 100 μM DHA and then stimulated with 50 μM LA for another 12 h after changing the medium. To investigate the effects of PPARγ, cells were pretreated with GW9662 for 2 h and then incubated for 12 h with 100 μM DHA, followed by stimulation with 50 μM LA for another 12 h.

2.4 Cell viability

Cell viability was determined with an MTT assay after a 12-h exposure to various concentrations (0–50 μM) of fatty acids. Five milligrams/milliliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Chemicon, Temecula, CA, USA) was added to 0.1 mL of cell suspension for 4 h, and the formazan that formed was then dissolved in isopropanol. The OD was measured with a plate reader at 570 nm.

2.5 Total RNA extraction and RT-PCR

Total RNA (1 μg) was prepared from ARPE-19 cells, and first-strand cDNAs were synthesized with an oligo-dT-primed Moloney murine leukemia virus reverse transcriptase (Invitrogen-Gibco, Carlsbad, CA, USA). For PCR, 1 μL of the cDNA mixture was added to a 50 μL PCR reaction mixture consisting of 5 μL of 10× PCR buffer, 2.5 pmol dNTP, 5 pmol paired primers, 1.25 U Taq polymerase (Promega, Madison, WI, USA), and ultrapure water. The following primers were used for the amplification reaction: for MCP-1, forward primer 5′-GCTCATAGCCACCTT CATTC-3′, reverse primer 5′-GTCTTTGGGAGTTTGGTTTC-3′; for PPARγ,
ARPE-19 cells were seeded per well on and www.mnf-journal.com + 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 10 g at 4 °C for 10 min. The levels of g-actin were determined by the bicinechonic acid (BCA) method (Pierce, Rockford, IL, USA). Samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with MCP-1 antibody. Then the membrane was hybridized with horseradish peroxidase conjugated secondary antibody and visualized by chemiluminescence (GE Healthcare). The density of blots was determined using image-analysis software (Photoshop, ver.7.0; Adobe, San Jose, CA), and the results were standardized against the intensity of rat b-actin band.

2.6 Western blot analysis

Cells were harvested, washed twice with ice-cold PBS, and resuspended in PBS containing 0.1 mM PMSF. The suspension was lysed by three cycles of freezing and thawing. The cytosolic fractions were obtained from the supernatant after centrifugation at 12 000 × g for 20 min. The nuclear fractions were washed twice in buffer A and resuspended in the same buffer. Nuclei were extracted for 1 h at 4 °C and centrifuged at 100 000 × g for 45 min. Supernatants were dialyzed in buffer C and stored at −80 °C. The protein concentration was determined by the BCA method. Nuclear extracts were used for the EMSA using an NF-kB DNA-binding protein detection system kit (Pierce).

2.7 Measuring MCP-1 production by RPE cells

To examine the production of MCP-1 in the cell culture medium, supernatants were removed at the end of the treatment and centrifuged at 500 × g for 10 min. The levels of MCP-1 in the cell culture supernatants were measured with an ELISA kit (R&D Systems, Minneapolis, MN, USA).

2.8 Nuclear protein extracts and electrophoretic mobility shift assay (EMSA)

ARPE-19 cells were trypsinized and homogenized in buffer A. Nuclei and cytosolic fractions were obtained by centrifugation at 1000 × g for 20 min. The nuclear fractions were washed twice in buffer A and resuspended in the same buffer. Nuclei were extracted for 1 h at 4 °C and centrifuged at 100 000 × g for 45 min. Supernatants were dialyzed in buffer C and stored at −80°C. The protein concentration was determined by the BCA method. Nuclear extracts were used for the EMSA using an NF-kB DNA-binding protein detection system kit (Pierce).

2.9 Transcriptional assay of MCP-1 gene

MCP-1 promoter plasmid was a kind gift from Professor Tanimoto. The human MCP-1 promoter region from the genomic DNA of U937 cells was cloned by PCR using the primer pairs 5′-GCTGGAGGCGAGAGTGCGAG-3′ and 5′-TCTAGATTCTCTCTAGCTGT-3′, corresponding to nucleotide positions −932 to +60 relative to the transcription start site. The PCR products were cloned into pGL3-basic vector (Promega, Madison, WI, USA), and the sequence was confirmed. The 5′-serial and internal deletion mutants were generated by appropriate enzymatic digestion and ligation.

2.10 Transient transfection and luciferase assay

A total of 2–4 × 10^5 ARPE-19 cells were seeded per well on a 24-well tissue culture dish 1 day before transient transfection. Cells were transfected with serum-free DMEM containing 25 µL/mL of Lipofectamine 2000 reagent (Life, Carlsbad, CA, USA) and 10 µg/mL plasmid DNA including an NF-kB luciferase promoter construct or the empty vector (Clontech, San Diego, CA, USA). Four hours after transfection, cells were washed with PBS and incubated with DMEM/F-12 for 24 h. Luciferase activity was measured using the Luciferase Reporter Assay System (Promega, Madison, WI, USA) and a luminometer.

2.11 Trans-AM PPARγ transcription factor assay

The Trans-AM PPARγ Kit (Active Motif, Carlsbad, CA, USA) contains a 96-well plate on which an oligonucleotide containing a peroxisome proliferator response element (5′-AACCCTAAGGCCAACCGTG-3′) has been immobilized. The primary antibody used in the Trans-AM PPARγ Kit recognizes an accessible epitope on PPAR-γ protein upon DNA binding. To quantify PPAR-γ activation, 5 µg of nuclear extract was measured using the Trans-AM PPARγ Kit according to the manufacturer’s instructions.

2.12 Immunofluorescent detection of NF-κB p65

ARPE-19 cells were grown to 50–70% confluence on cover slips in 24-well plates (Corning, NY, USA). After treatment, cells were fixed with 100% ice-cold methanol, blocked with 25% nonimmune goat serum (Sigma-Aldrich), and then incubated for 30 min with rabbit anti-NF-κB p65 antibody.

2.13 Matrigel capillary tube formation assay

An assay on synthetic matrix (Matrigel; BD Bioscience, NJ, USA) was performed. Fifty microliters of Matrigel were plated into individual wells of a 96-well plate and then allowed
polymerrize at 37°C for 30–60 min. Monkey choroidal endothelial cells (RF6A) were removed from culture and re-suspended at a concentration of 5 × 10⁵ cells/mL in M199 containing 2% FBS. The conditioned medium of previously treated ARPE-19 cells was added to 100 μL of RF6A suspension and then plated in each well, followed by incubation for 8–12 h. Each chamber was photographed at a final magnification of 100×. Each concentration of control or test substance was assayed in triplicate.

2.14 Statistical analysis

The results are expressed as the mean ± SD. Data were analyzed using one-way analysis of variance followed by Bonferroni multiple comparison. A p-value of 0.05 or less was considered significant.

3 Results

3.1 Effects of ω-3 and ω-6 fatty acids on the expression of MCP-1 at the mRNA and protein levels in ARPE-19 cells

Exposure of cells to ω-6 fatty acids LA and AA at concentrations of 10, 25, or 50 μM for 12 h increased the expression of MCP-1 mRNA in a dose-dependent manner (p < 0.05 compared with the cells treated with vehicle; Fig. 1A–D), whereas exposure to the ω-3 fatty acids DHA and EPA (0–100 μM) had no effect on the expression of MCP-1 mRNA. The viability of ARPE-19 cells was not decreased when these cells were incubated with 50 μM LA and AA, or 100 μM DHA and EPA for 12 h (data not shown).

Similarly, exposure of cells to the ω-6 fatty acids LA and AA at concentrations of 10, 25, or 50 μM increased the synthesis of MCP-1 protein in a dose-dependent manner (p < 0.05 compared with the cells treated with vehicle), whereas exposure to the ω-3 fatty acids DHA and EPA had no effect on the expression of MCP-1 protein. LA is also more potent than AA in stimulating the synthesis of the MCP-1 protein (Fig. 1E–H).

3.2 Effects of ω-3 and ω-6 fatty acids on the release of the MCP-1 protein in the culture medium of ARPE-19 cells

We determined the release of the MCP-1 protein in the culture medium of ARPE-19 cells with an ELISA. Consistent with the expression of MCP-1 protein in ARPE-19 cells, exposure of cells to LA and AA at 10, 25, or 50 μM for 12 h increased the release of the MCP-1 protein in the culture medium of ARPE-19 cells in a dose-dependent manner (Fig. 2A and B), whereas exposure to DHA and EPA (0–100 μM) had no effect on the release of the MCP-1 protein in the culture medium of ARPE-19 cells (Fig. 2C and D).

3.3 Luciferase assay using 5′-serial and internal deletions of MCP-1 promoter constructs

The vector constructs used were the human MCP-1 promoter region up to −932 bp, including the AP-1, NF-κB, and STAT3 motifs, and its deletion mutations. LA and AA at 50 μM increased the transcriptional activity from the −921 bp construct, whereas 100 μM DHA and EPA had no effect on the transcriptional activity of this construct. The transcriptional activity remained increased after stimulation with 50 μM LA and AA when the 5′-deletion construct up to −145 was used for transfection. The deletion constructs up to −79 showed significantly decreased responses to LA and AA. When the construct was deleted up to −38, the LA and AA response remained decreased (p < 0.05 in paired comparisons with MCP-1 promoter constructs without internal deletions; Fig. 3A). These results indicated the presence of motifs responsive to the ω-6 fatty acids LA and AA in the region spanning from −145 to −80, which contains the AP-1 and NF-κB motifs.

3.4 Effects of ω-3 and ω-6 fatty acids on NF-κB activation in ARPE-19 cells

The NF-κB/DNA-binding activity was measured using EMSA. We found that exposure of cells to the ω-6 fatty acids LA and AA at 50 μM for 8 h resulted in increased NF-κB/DNA-binding activity. The ω-3 fatty acids DHA and EPA (100 μM) had no effect on NF-κB/DNA-binding activity (Fig. 3B).

We further investigated whether the increase in NF-κB/DNA binding mediated by LA and AA corresponded with an increase in NF-κB-dependent gene transcription. We found that the exposure of ARPE-19 cells to 50 μM LA and AA also induced an increase in luciferase activity in the cells transfected with the NF-κB-luciferase reporter construct, but not in those transfected with the empty vector (p < 0.05 compared with cells treated with vehicle) (Fig. 3C).

3.5 DHA-inhibited LA-induced expression of MCP-1 mRNA and protein in ARPE-19 cells, and MCP-1 secretion in the culture media

We next determined the effects of DHA on LA-induced MCP-1 expression in ARPE-19 cells. Pretreatment with DHA at concentrations of 50 or 100 μM inhibited the expression of MCP-1 at the mRNA and protein levels in a dose-dependent fashion in LA-stimulated ARPE-19 cells (p < 0.05 compared with cells treated with 50 μM LA only) (Fig. 4A and B).

Similarity, pretreatment with 50 or 100 μM DHA significantly inhibited the release of MCP-1 in the culture medium.
of ARPE-19 cells stimulated with 50 μM LA (p < 0.05 compared with cells treated with 50 μM LA only) (Fig. 4C).

**3.6 DHA-inhibited LA-induced MCP-1 expression by inhibiting NF-κB activation**

EMSA showed that pretreatment with 50 and 100 μM DHA decreased the NF-κB/DNA-binding ability in LA-stimulated ARPE-19 cells (Fig. 4D). Similarly, pretreatment with 50 and 100 μM DHA decreased the luciferase activity in a dose-dependent manner in LA-stimulated ARPE-19 cells transfected with the NF-κB-luciferase reporter construct (p < 0.05 compared with cells treated with 50 μM LA only) (Fig. 4E).

**3.7 Effects of LA and DHA on PPARγ protein expression and activation in ARPE-19 cells**

Exposure of ARPE-19 cells to 100 μM DHA and 5 μM troglitazone increased PPARγ protein expression (p < 0.05 compared with cells treated with vehicle; n = 3).
compared with cells treated with vehicle), whereas exposure to LA had no effect on PPARγ protein expression (Fig. 5A). Furthermore, we showed that DHA induced an approximately threefold increase in PPARγ activation compared with vehicle using a Trans-AM PPARγ transcription factor assay (*p < 0.05 compared with cells treated with vehicle; n = 3).

Figure 2. Effects of LA, AA, DHA, and EPA on the secretion of MCP-1 in the culture medium of ARPE-19 cells. Cells were treated with vehicle or increasing concentrations (10, 25, or 50 µM) of LA (A), AA (B), or 25, 50, or 100 µM DHA (C) or EPA (D) for 12 h or with TNF-α as a positive control. The culture medium in each group was collected and analyzed by ELISA. Data are expressed as the mean ± SD (*p < 0.05 compared with cells treated with vehicle; n = 3).

Figure 3. Effects of LA, AA, DHA, and EPA on luciferase assay using internal deletions of MCP-1 promoter constructs, NF-κB-binding ability, and NF-κB-dependent transcriptional activity in ARPE-19 cells. (A) The response to 50 µM LA and AA significantly decreased when further deleting the construct from −145 to −80, which contains the AP-1 d and κB motifs (*p < 0.05 in paired comparisons with MCP-1 promoter constructs without internal deletions). (B) NF-κB-binding ability was analyzed by electrophoretic mobility shift assay. (C) NF-κB-dependent transcriptional activity was determined using a luciferase assay. None of the fatty acids influenced the luciferase activity in cells transfected with an enhancerless construct (pTAL-luc). Specific activity is expressed as units/microgram of protein. Data are expressed as the mean ± SD (*p < 0.05 compared with cells treated with vehicle; n = 3).
3.8 PPAR-γ was involved in DHA-inhibited LA-induced NF-κB activation in ARPE-19 cells

Pretreatment with the PPARγ inhibitor GW9662 abolished the inhibitory effects of DHA on LA-induced MCP-1 protein expression in a dose-dependent manner (*P < 0.05 compared with the cells treated with DHA and LA) (Fig. 5C). In addition, GW9662 could also recover the loss of NF-κB/DNA-binding ability inhibited by DHA in LA-stimulated ARPE-19 cells (Fig. 5D). Similarly, GW9662 reversed the decrease in NF-κB-dependent transcriptional activity that was inhibited by DHA in a dose-dependent manner (*P < 0.05 compared with cells treated with DHA and LA) (Fig. 5E).

3.9 DHA-inhibited LA-induced NF-κB activation was mediated by the activation of PPARγ, which then inhibited IκB degradation and NF-κB p65 translocation in ARPE-19 cells

The levels of IκB were significantly reduced in ARPE-19 cells after 30 min of LA stimulation. Treatment with 100 μM DHA significantly reversed the reduction in IκB at 30 min after stimulation with 50 μM LA (*P < 0.05 in paired comparisons) (Fig. 6A). However, pretreatment with GW9662 resumed the decrease in the levels of IκB in LA-stimulated ARPE-19 cells treated with DHA (*P < 0.05 compared with cells treated with DHA and LA) (Fig. 6B). Meanwhile, pretreatment with
10 or 20 μM GW9662 recovered the amount of NF-κB p65 in the nucleus in a dose-dependent manner in LA-stimulated ARPE-19 cells treated with DHA (\(p < 0.05\) compared with cells treated with DHA and LA) (Fig. 6C).

Immunohistochemistry staining showed that NF-κB p65 was located in the cytoplasm in normal control and DHA-treated ARPE-19 cells. NF-κB p65 was found to translocate into the nucleus after stimulation with LA. DHA markedly inhibited the translocation of NF-κB p65 into the nucleus in LA-stimulated ARPE-19 cells. However, pretreatment with 10 or 20 μM GW9662 recovered the translocation of NF-κB p65 into the nucleus in LA-stimulated ARPE-19 cells treated with DHA (Fig. 6D).

3.10 The inhibition of the LA-induced release of MCP-1 protein in the culture medium and on the endothelial cell tube formation by DHA was mediated by the activation of PPARγ

ELISA showed that LA induced the release of MCP-1 protein in the culture medium of ARPE-19 cells, whereas DHA and
Figure 6. DHA-inhibited LA-induced NF-κB activation is mediated by PPARγ activation, which inhibits IκB degradation and NF-κB p65 translocation in ARPE-19 cells. (A) ARPE-19 cells were treated with 100 μM DHA for 12 h. After changing the medium, the cells were treated with 50 μM LA for the indicated times. Cytoplasmic IκB protein was determined by Western blot analysis. Data are expressed as the mean ± SD (*p < 0.05 in paired comparisons, n = 3). (B) ARPE-19 cells were pretreated with GW9662 for 2 h and then exposed to 100 μM DHA for 12 h, followed by stimulation with 50 μM LA for 30 min. Cytoplasmic IκB protein and (C) nuclear NF-κB p65 were determined by Western blot analysis (*p < 0.05 compared with cells treated with DHA and LA; n = 3). (D) Immunohistochemical staining of NF-κB p65. NF-κB p65 was located in the cytoplasm in normal or DHA-treated ARPE-19 cells (upper right and middle). In LA-stimulated cells, NF-κB p65 was translocated into the nucleus (upper left). The translocation of NF-κB p65 into the nucleus was markedly inhibited by treatment with DHA in LA-stimulated ARPE-19 cells (lower right). However, pretreatment with 10 or 20 μM GW9662 restored the translocation of NF-κB p65 into the nucleus in LA-stimulated ARPE-19 cells treated with DHA (lower middle and left).

4 Discussion

Selected dietary fatty acids can modulate the inflammatory response in numerous tissues, including RPE. Among the different dietary fatty acids, LA and DHA may play critical roles in the induction and alteration of RPE cell metabolism. LA is present in high concentrations in corn, soy, sunflower, and safflower oils [24]. It is estimated that LA provides approximately 7–8% of the average dietary energy intake. DHA is present mainly in fish and is highly concentrated in the POS of the retina [25]. In this study, we found that the ω-6 PUFAs LA and AA could induce increases in the mRNA and protein levels of MCP-1 in ARPE-19 cells and then promote
I.-M. Fang et al.


Figure 7. The inhibitory effects of DHA on LA-induced MCP-1 production and endothelial cell tube formation were mediated via PPARγ activation. (A) The concentration of MCP-1 in the culture medium was measured by ELISA (*p < 0.05 compared with cells treated with vehicle; n = 3). (B) Photomicrograph of RF6A tube formation. The culture medium of ARPE-19 cells stimulated with 50 μM LA could induce tube formation (upper middle). Culture medium from cells stimulated with vehicle or 100 μM DHA could not induce tube formation (upper right and left). LA-induced tube formation was inhibited by pretreatment with DHA (lower left). However, pretreatment with the PPARγ inhibitor GW9662 restored tube formation in LA-stimulated ARPE-19 cells treated with DHA (lower right). Original magnification, ×100.

the release of the MCP-1 protein, which may contribute to the formation of CNV. LA-induced MCP-1 expression in ARPE-19 cells is mainly mediated by the activation of NF-κB. Furthermore, the ω-3 PUFA DHA abolished LA-induced activation of NF-κB and MCP-1 expression in ARPE-19 cells. The inhibitory effect of DHA was achieved by the activation of PPARγ, which then inhibited IκB degradation and p65 translocation in LA-stimulated ARPE-19 cells. Our findings might provide the molecular mechanisms for the beneficial effects of the increased consumption of DHA and the reduced consumption of LA on AMD.

ω-3 and ω-6 PUFA s are metabolically and functionally distinct and often have opposing physiological effects. It is generally accepted that ω-6 PUFA s are proinflammatory and show disease-propagation effects, whereas ω-3 PUFA s are anti-inflammatory and show disease-ameliorating effects [26]. LA and its metabolite s possess potent proinflammatory activity. Matesanz et al. demonstrated that LA increases the secretion of IL-8, MCP-1, and prostaglandins, which enhanced monocyte chemotaxis and adhesion in human aortic endothelial cells [27]. In contrast, DHA and EPA inhibited the production of proinflammatory cytokines in response to injury in several cell types in vitro and in vivo [28,29]. Moreover, ω-3 PUFA s such as EPA and DHA can antagonize AA metabolism and suppress the production of proinflammatory eicosanoids [30]. Our results are in agreement with those of previous studies in showing that LA induced the expression of the proinflammatory chemokine MCP-1, whereas DHA inhibited the expression of MCP-1 in LA-stimulated RPE cells.

The ability of ω-6 PUFA s such as LA and AA to activate NF-κB and induce NF-κB-dependent gene expression had been reported in several cell types [31,32]. We had previously showed that LA induced the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase II via p42/44 mitogen-activated protein kinase and NF-κB in RPE cells [33]. The promoter of the MCP-1 gene contains NF-κB-binding sites, which have been shown to be important for their expression [34]. Lee et al. demonstrated that LA-induced MCP-1 gene expression in human microvascular endothelial cells through NF-κB-related mechanisms [35]. In contrast, several studies indicated that the ability of ω-3 PUFA s to inhibit inflammation is attributable in part to the suppression of NF-κB activation [36]. In this study, we extended the scope by demonstrating that LA could activate NF-κB and induce the expression of the NF-κB-dependent gene MCP-1 in RPE cells. More importantly,
DHA could attenuate LA-induced NF-κB activation in RPE cells.

It is generally accepted that PPARγ plays a protective role because many PPARγ ligands can effectively reduce inflammatory processes in vitro and in vivo [37, 38]. The anti-inflammatory activity of PPARγ ligands has partly been attributed to the inhibition of the NF-κB pathway. DHA and EPA are known as natural ligands of PPARγ. Marion-Letellier et al. demonstrated that DHA and EPA decreased iNOS expression in intestinal-like Caco-2 cells by activation of PPARγ and IκB [39]. In the present study, we demonstrated that DHA could induce the expression and activation of PPARγ in ARPE-19 cells. In addition, the PPARγ antagonist GW9662 negate the inhibitory effect of DHA in a dose-dependent manner, indicating that PPARγ participated in the inhibition of DHA on LA-induced activation of NF-κB and MCP-1 expression in ARPE-19 cells. Furthermore, we explored the possible mechanism underlying this inhibition. Several studies have shown that PPARγ inhibited NF-κB signaling by the inhibition of IκBα expression, phosphorylation, and subsequent degradation, as well as p65 nuclear translocation [40, 41]. In this study, we found that pretreatment with DHA decreased the degradation of cytoplasmic IκB and thus reduced the translocation of p65 into the nucleus in LA-stimulated ARPE-19 cells. However, treatment with the PPARγ antagonist GW9662 restored the degradation of IκB and the translocation of p65 in LA-stimulated ARPE-19 cells. Taken together, these findings indicate that the DHA-inhibited LA-induced activation of NF-κB was mediated through PPARγ activation, which then inhibited IκB degradation and p65 translocation.

There are several limitations to this study. One noteworthy caveat is that, because this was an in vitro study, we investigated the effects of ω-3 and ω-6 fatty acids by individually adding them to cell cultures. However, in vivo situations are far more complex: because ω-3 and ω-6 fatty acids share the same series of conversion enzymes, a competition exists between ω-3 and ω-6 fatty acids for conversion to metabolites. Moreover, the cells are removed from their natural surroundings, resulting difficulties in the estimation of the effects of local tissue factors on the metabolism of fatty acids or on the expression of inflammatory genes in RPE. In addition, we used 100 μM DHA and 50 μM LA to treated ARPE-19 cells in vitro. Under physiological condition, it is generally hard to reach a similar high dose of DHA and LA only by an appropriate DHA and LA consumption in daily diet. Despite these limitations, this study provided useful information about the effects of ω-3 and ω-6 fatty acids on the expression of the proinflammatory gene MCP-1 in RPE under carefully controlled cell conditions.

In conclusion, we demonstrated DHA-reduced LA-induced MCP-1 expression via a PPARγ and NF-κB-dependent pathway in ARPE-19 cells. Figure 8 depicts a model for the signaling mechanisms in MCP-1 expression between LA and DHA in ARPE-19 cells. These results might provide the molecular mechanisms for the beneficial effects of the increased consumption of DHA and the reduced consumption of LA on AMD.
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The authors have declared no conflict of interest.

5 References


