Food and Chemical Toxicology 134 (2019) 110811

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Docosahexaenoic acid inhibits TNFα-induced ICAM-1 expression by activating PPARα and autophagy in human endothelial cells

Hung-Chih Lin[a,1], Chong-Kuei Lii[b,c,1], Ai-Hsuan Lin[b], Chien-Chun Li[d,e], Chia-Han Tsai[d], Shiuan-Kai Pan[b], Ya-Chen Yang[c], Chin-Shiu Huang[c], Latif Reshi[b], Haw-Wen Chen[b,*]

[a] Division of Neonatology, College of Medicine and Department of Pediatrics, Children's Hospital of China Medical University and China Medical University Hospital, Taichung, Taiwan
[b] Department of Nutrition, China Medical University, Taichung, Taiwan
[c] Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan
[d] Department of Nutrition, Chung Shan Medical University, Taichung, Taiwan
[e] Department of Nutrition, Chung Shan Medical University Hospital, Taichung, Taiwan

ARTICLE INFO

Keywords:
Autophagy
Docosahexaenoic acid (DHA)
Intercellular adhesion molecule 1 (ICAM-1)
Peroxisome proliferator-activated receptor alpha (PPARα)
Tumor necrosis factor-alpha (TNFα)

ABSTRACT

Inflammation plays a key role in the development of cardiovascular disease (CVD), and docosahexaenoic acid (DHA) is recognized to fight against CVD. PPARα belongs to the nuclear hormone receptor superfamily and can interfere with inflammatory processes. Autophagy can degrade inflammasome proteins and counteract inflammation. Overexpression of intercellular adhesion molecule (ICAM) 1 in endothelial cells contributes to monocyte migration into the vascular intima. Here we investigated the mechanisms by which DHA inhibits TNFα-induced ICAM-1 expression in EA. hy926 endothelial cells. DHA markedly activated PPARα and suppressed TNFα-induced ICAM-1 expression, ICAM-1 promoter activity, p65 nuclear translocation, NFκB and DNA binding activity, and THP-1 cell adhesion. PPARα knockdown abolished the ability of DHA to inhibit TNFα-induced ICAM-1 expression and THP-1 cell adhesion. The PPARα antagonist GW6471 reversed the inhibitory effect of DHA on TNFα-induced ICAM-1 expression, p65 nuclear translocation, NFκB and DNA binding activity, and THP-1 cell adhesion. DHA significantly activated autophagy as evidenced by the formation of autophagosomes and increased LC3II protein expression. By contrast, wortmannin, which inhibits autophagy, abrogated DHA-induced autophagy and the inhibition of TNFα-induced ICAM-1 protein expression by DHA. Our results suggest that DHA likely inhibits TNFα-induced ICAM-1 expression by activating PPARα and autophagy.

1. Introduction

Cardiovascular disease (CVD) is a leading cause of death in Taiwan (Taiwan Health and Welfare Report, 2017), similar to developed countries (Hoyert, 2012). CVD is intimately associated with atherosclerosis, which is a chronic inflammatory disease of the vasculature characterized by infiltration of leukocytes, deposition of lipids, and thickening of the vascular wall in response to inflammatory cytokines (Lusis, 2000). Inflammation is now widely accepted as an early event in the development of atherosclerosis (Libby, 2002). Leukocyte recruitment is a multistep process that is mainly mediated by cellular adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1). Tumor necrosis factor-alpha (TNFα), a proinflammatory cytokine, is generally present in atherosclerotic lesions and triggers the expression of ICAM-1, which depends on the activation of nuclear factor-κB (NFκB) (Oh et al., 2010). Activation of NFκB is determined by its nuclear translocation. For activation to occur, the inhibitory kappa B (IκB) family is phosphorylated by the IκB kinase (IKK) complex. The IκB proteins undergo

Abbreviations: ATG, autophagy-related; BCECF-AM, 2′,7′-bis-(Carboxyethyl)-5(6′)-carboxyfluorescein acetoxymethyl ester; BCP, 1-bromo-3-chloropropane; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EMSA, electrophoretic mobility shift assay; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphatedehydrogenase; ICAM-1, intercellular adhesion molecule 1; IκB, inhibitory kappa B; IKK, IκB kinase; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFκB, nuclear factor-κB; PI3K, phosphoinositide 3-kinase; PPARα, peroxisome proliferator-activated receptor alpha; PPAR-KO, PPARα knockout; PPRE, PPAR response elements; ROS, reactive oxygen species; RXR, retinoic X receptor; SEAP, secreted embryonic alkaline phosphatase; TNFα, tumor necrosis factor-alpha; VCAM-1, vascular adhesion molecule 1

* Corresponding author.
E-mail address: chenhw@mail.cmu.edu.tw (H.-W. Chen).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fct.2019.110811
Received 5 June 2019; Received in revised form 17 August 2019; Accepted 6 September 2019
Available online 06 September 2019
0278-6915/

proteasome degradation when exposed to inflammatory stimulation (Karin and Delhase, 2000).

Evidence from epidemiologic studies on Greenland Inuits performed in the 1970s and subsequent human studies suggests an inverse correlation between the consumption of n-3 PUFAs and mortality associated with CVD (Jump et al., 2012). The CVD protective effect of n-3 PUFAs is attributed to their hypolipemic, anti-inflammatory, and anti-arrhythmic activities (Jump et al., 2012). Therefore, national heart associations and government agencies have recommended increases in the ingestion of fatty fish or n-3 PUFAs to fight against CVD. Docosahexaenoic acid (DHA) has been shown to have anti-inflammatory activity relevant to CVD, such as inhibiting TNFα-induced ICAM-1 expression in endothelial cells and monocyte adhesion (Liu et al., 2016; Yang et al., 2013).

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily, which consists of PPARα, PPARβ/δ, and PPARγ. Their natural activating ligands comprise lipid-derived substrates and phytochemicals (Penumetcha and Santanam, 2012). The lipid-derived substrates include linoleic acid, nitrolinoleic acid, DHA, and conjugated linoleic acid, and the phytochemicals include isoflavones, flavonoids, quercetin, 2′-hydroxy chalcone, and rosmarinic acid. PPARα expression is relatively high in hepatocytes, endothelial cells, and lymphocytes (Wan et al., 2000; Inoue et al., 2001; Heneka and Landreth, 2007). In the liver, PPARα plays a critical role in fatty acid oxidation and has a potential role in the oxidant/antioxidant pathway (Lefebvre et al., 2006; Rigamonti et al., 2008). In addition to regulating lipid metabolism, PPARα is important for anti-inflammation. It has been reported that the PPARα agonist fenofibrate inhibits IL-1β-induced nuclear translocation of NFκB and the expression of inflammatory genes such as IL-6, IL-8, and GM-CSF (Okamoto et al., 2005). Regarding the relationship with PPARα, DHA has been shown to bind with high affinity to PPARα and RXR by use of the computational method of molecular dynamic simulation (Gani and Sylte, 2008). Moreover, DHA is implicated as a PPARα ligand and inhibits NFκB binding activity (Adkins and Kelley, 2010).

Autophagy is a key intracellular process that targets defective organelles to the lysosomes for degradation (Mao et al., 2016). Autophagy is highly controlled by autophagy-related (*ATG*) genes, which encode proteins essential for autophagosome formation, cargo degradation, and reuse of degraded materials contained in autophagosomes (Levine and Yuan, 2005; He and Klionsky, 2009). The best characterized proteins are *Atg 8*/LC3, *Atg 7*, and *Atg 6*/Beclin-1 with the class III phosphoinositide 3-kinase (PI3K) complex (Mao et al., 2016). It has been reported that the anti-inflammatory effect of several food components on the vascular endothelium is attributed to their action on inducing autophagy (Huang et al., 2017). For example, DHA has been demonstrated to induce autophagy through p53/AMPK/mTOR signaling in human cancer cells harboring wild-type p53 (Jing et al., 2011).

Several working mechanisms have been proposed for the anti-inflammatory potential of DHA. These include inhibition of the IKK/NFκB pathway, induction of the Nrf2/HO-1 pathway, interference with TAB2 interaction with TAK1/TAB1, and downregulation of ERK-dependent Egr-1 expression. It is also known that DHA is a ligand for PPARα and an activator of autophagy. Therefore, the present study was undertaken to clarify the roles of PPARα and autophagy in the inhibition by DHA of TNFα-induced ICAM-1 expression.

2. Materials and methods

2.1. Chemicals

DMEM, RPMI-1640, OPTI-MEM, Ham's F–12K medium, 0.25% trypsin-EDTA, and penicillin/streptomycin were from GIBCO (Grand Island, NY); fetal bovine serum (FBS) was from HyClone (Logan, UT); DHA was from Cayman Chemical (Ann Arbor, MI); 2′,7′-bis-(carboxyethyl)-5 (6′)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was from Calbiochem (Darmstadt, Germany); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), recombinant human TNFα, clofibrate, GW6471, wortmannin, and antibody against β-actin were from Sigma-Aldrich (St. Louis, MO); antibody against PPARα was from Santa Cruz Biotechnology (Santa Cruz, CA); antibody against p65 was from BD Bioscience (San Jose, CA); antibodies against ICAM-1 and LC3I/II were from Cell Signaling Technology (Boston, MA); antibody against GAPDH was from Millipore (Billerica, MA); and molecular biology grade 1-bromo-3-chloropropane (BCP) was from Molecular Research Center Inc. (Cincinnati, OH).

2.2. Cell cultures

EA.hy926 was cultured in DMEM supplemented with 1.5 g/L $NaHCO_3$, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% FBS at 37 °C in a 5% CO_2 humidified incubator. The human leukemic cell line THP-1 was obtained from Bioresources Collection and Research Center (BCRC, Hsinshu, Taiwan). The THP-1 cells were cultured in T-75 tissue culture flasks in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 humidified incubator. Chinese hamster ovary cell line CHO–K1 was cultured in Ham's F–12K medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin.

2.3. Fatty acid preparation

Sodium salt of DHA was prepared and complexed with fatty acid-free bovine serum albumin at a 6:1 M ratio before addition to the culture medium. At the same time, 0.1% butylated hydroxytoluene and 20 μM α-tocopheryl succinate were added to the culture medium to prevent lipid peroxidation.

2.4. Western blot analysis

After each experiment, cells were washed twice with cold PBS and were harvested in 150 μL of lysis buffer (10 mM Tris-HCl, pH 8.0, 0.35% Triton X-100, 320 mM sucrose, 5 mM EDTA, 1 mM PMSF, 1 μg/L leupeptin, 1 μg/L aprotinin, and 2 mM dithiothreitol). Cell homogenates were centrifuged at 10,000 × *g* for 15 min at 4 °C. The resulting supernatant was used as a cellular protein for Western blotanalysis. The total protein was analyzed by use of the Coomassie Plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL). Equal amounts of cellular proteins were electrophoresed in a SDS-polyacrylamide gel, and then proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Nonspecific binding sites on the membranes were blocked with 5% nonfat milk in 15 mM Tris/150 mM NaCl buffer (pH 7.4) at room temperature for 2 h. Membranes were probed with antibodies followed by hybridizing with the secondary antibody labeled with horseradish peroxidase. The bands were visualized by using the Luminata™ Western HRP substrate (Millipore, Billerica, MA) and scanned by a luminescent image analyzer (LAS-4000, FUJIFILM, Japan).

2.5. RNA isolation and RT-PCR

Total RNA of EA. hy926 cells was extracted by using TRIzol reagent (Thermo Fisher, Carlsbad, CA). A total of 0.2 μg RNA was 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 $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 2.5 mM oligo (dT), and 40 U of RNase inhibitor. PCR amplification was conducted in a thermocycle in a reaction volume of 50 μL containing 20 μL of cDNA, BioTaq PCR buffer, 4 mM $MgCl_2$, 1 U of BioTaq DNA polymerase (BioLine), and 6 pmol forward and reverse primers. Oligonucleotide primers were as follows: ICAM-1 (forward, 5′-TGAAGGCCACCCCAGA

GGACAAC-3'; reverse, 5′-CCCATTATGACTGCGGCTGCTACC-3′), PPARα (forward, 5′-CCAGTATTTAGGAAGCTGTCC-3'; reverse, 5′-AAG TTCTTCAAGTAGGCCTCG-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5′-CCATCACCATCTTCCAGGAG-3'; reverse, 5′-CCTGCTTCACCACCTTCTTG-3′). Amplification of ICAM-1 and GAPDH was achieved when samples were heated to 95 °C for 5 min and then immediately cycled 32 times through a 40 s denaturing step at 94 °C, a 1 min annealing step at 52 °C, and a 1 min elongation step at 72 °C, and then a 10 min final extension at 72 °C. Amplification of PPARα was achieved when samples were heated to 95 °C for 5 min and then immediately cycled 29 times through a 30 s denaturing step at 94 °C, a 50 s annealing step at 54 °C, and a 50 s elongation step at 72 °C, and then a 10 min final extension at 72 °C. The GAPDH cDNA level was used as the internal standard.

2.6. RNA interference by small hairpin RNA of PPARα

Lentiviral infection was performed according to the method of a previous study (Tang et al., 2011). One sequence targeting human PPARα mRNA was chosen from the National RNAi Core facility platform, Taiwan. RNAi clones were identified by their unique number assigned by the RNAi Consortium (TRCN) as follows: TRCN0000001665 (responding sequence: GAACAGAAACAAATGC CAG) was used for shPPARα targeted to PPARα and TRCN00000772246 (responding sequence: CAAATCACAGAATCGTCG TAT) was used for vector control targeted to luciferase. Briefly, EA. hy926 cells (0.3×10^6) were plated on 6-cm plastic culture dishes in DMEM supplemented with 1.5 g/L sodium bicarbonate, penicillin 100 U/mL, streptomycin 100 μg/mL, and 10% FBS. After 24 h of attachment, the cells were infected with packaged lentiviruses 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. The lentivirus treatment of EA. hy926 cells was multiplicity of infection (MOI) of 3.

2.7. Transient transfections and reporter gene assay

The chimeric receptor construct pBK-CMV-Gal4-rPPARα-ligand-binding-domain (Gal4-rPPARαLBD) and the reporter gene pBK-CMV-$(UAS)_4$-tk-alkaline phosphatase (AP) were gifts from Dr. C. Y. Chao, Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan. A reporter gene assay was used to measure PPARα activation via release of secreted embryonic alkaline phosphatase (SEAP). Cells were transiently transfected with 1 μg of Gal4-rPPARαLBD plasmid and 0.5 μg of pBK-CMV-$(UAS)_4$-tk-alkaline phosphatase (AP) plasmid by using 8 μL of nanofectin in OPTI-MEM medium for 12 h. All transfection experiments were performed by using nanofectin reagent (PAA, Pasching, Austria) according to the manufacturer's instructions. After transfection, cells were changed to Ham's F–12K medium and treated with 100 μM DHA or 100 μM clofibrate as a positive control at 37 °C for 24 h. After incubation, the medium was collected, SEAP substrate CDP-Star® was added (Clontech Laboratories, Palo Alto, CA), and activity was detected by using Great EscAPeTM SEAP chemiluminescence Kit 2.0 (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's instructions. Luciferase activity was measured in a Synergy™ HT multi-mode microplate reader (Bio-Tek Instruments, Winooski, VT) with an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

The ICAM-1 promoter-luciferase construct (pIC339) containing NFκB (−187/-178), AP-1 (−48/-41), and Sp-1 (−59/-53, −206/-201) binding sites (van de Stolpe et al., 1996) was a gift from Dr. P. T. van der Saag (Hubrecht Laboratory, Utrecht, The Netherlands). Cells were transiently transfected with 0.4 μg of pIC339 plasmid and 0.2 μg of β-galactosidase plasmid by using 1 μL of nanofectin in OPTI-MEM medium for 24 h. After transfection, cells were changed to DMEM and treated with or without 8 μM GW6471 for 15 min followed by incubation with 100 μM DHA for 16 h before being challenged with 1 ng/mL TNFα for another 6 h. Activity of luciferase was measured by using a Luciferase Assay Kit (Promega, Madison, WI) in a Synergy™ HT multi-mode microplate reader (Bio-Tek Instruments, Winooski, VT). The luciferase activity of each sample was corrected on the basis of β-galactosidase activity, which was measured at 420 nm with O-nitrophenyl-beta-D-galactopyranoside as a substrate.

2.8. Nuclear extract preparation

After treatment, cells were washed twice with cold PBS and scraped from the dishes with 1 mL of PBS. Cell homogenates were centrifuged at $2000 \times g$ for 5 min. The supernatant was discarded, and the cell pellet was allowed to swell on ice for 15 min after addition of 200 μL of hypotonic buffer (10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.5% NP-40, 4 μg/mL leupeptin, 20 μg/mL aprotinin, and 0.2 mM PMSF). After centrifugation at $6000 \times g$ for 15 min, pellets containing crude nuclei were resuspended in 50 μL of hypertonic buffer (10 mM HEPES, 400 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 0.2 mM PMSF, and 10% glycerol) at 4 °C for 30 min followed by centrifugation at $10{,}000 \times g$ for 20 min. The supernatant containing the nuclear protein was collected and stored at −80 °C for Western blot analysis and DNA binding activity assays.

2.9. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed according to our previous study (Cheng et al., 2004). The LightShift Chemiluminescent EMSA kit and synthetic biotin-labeled double-stranded NFκB consensus oligonucleotides (forward, 5′-AGTTGAGGG GACTTTCCCAGGC-3'; reverse, 5′-GCCTGGGAAAGTCCCCTCAACT-3′) were used to measure the NFκB nuclear protein-DNA binding activity. Nuclear extractable proteins (12 μg), poly (dI-dC), and biotin-labeled double-stranded NFκB oligonucleotides were mixed with the binding buffer (to a final volume of 20 μL) and were incubated at 27 °C for 30 min. The unlabeled and a mutant double-stranded NFκB oligonucleotide (5′-AGTTGAGGCGACTTTCCCAGGC-3′) were used to confirm the protein binding specificity, respectively. These oligonucleotide primers were synthesized by MDBio Inc. (Taipei, Taiwan).

2.10. Monocyte adhesion assay

EA.hy926 cells in 12-well plates were allowed to grow to 80% confluence and were then pretreated with or without 8 μM GW6471 for 15 min and then with 100 μM DHA for 16 h before being challenged with 1 ng/mL TNFα for an additional 6 h. The role of PPARα in DHA inhibition of TNFα-induced monocyte adhesion was studied by using shPPARα. A total of 1×10^6 THP-1 cells labeled with BCECF-AM were added to each well, and the cells were co-incubated with EA. hy926 cells for 30 min (Lei et al., 2008). The cells were washed and filled with cell culture medium, and the plates were sealed, inverted, and centrifuged at $100 \times g$ for 5 min to remove nonadherent THP-1. Bound THP-1 cells were lysed in a 1% SDS solution, and the fluorescence intensity was determined in a fluoroscan ELISA reader (FLX800, Bio-Tek, Winooski, VT) with excitation and emission wavelengths of 480 and 520 nm, respectively.

2.11. Autophagy immunofluorescence detection

Detection of autophagic flux was performed by using the Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). EA. hy926 cells (0.4×10^6) were plated onto 6-cm plastic culture dishes in DMEM and allowed to reach 70% confluence before transduction with

the RFP-GFP-LC3B reagent for 16 h. Afterward, 100 μM DHA was added to the cells and the cells were incubated for another 16 h. Chloroquine diphosphate was added to the cells and incubated for 1 h as a positive control. After the experiment, the cells were harvested with 0.025% trypsin, washed with warm PBS, and detected by using flow cytometry (FASCSCalibur, BD Bioscience). The parameters of FSC and SSC were adjusted according to blank, and threshold settings of FL1 PMT (GFP: Green-515-545 nm), FL3 PMT (RFP: Red- > 650 nm) based on control cells (GFP/RFP < 5%) and chloroquine-treated cells (GFP/RFP > 15%). Samples were run through the flow cytometer until 10,000 gated events were collected and the RFP-GFP-LC3B-targeted cells were detected and quantified.

2.12. Statistical analysis

Data were analyzed by using analysis of variance (SAS Institute, Cary, NC). The significance of the difference among mean values was determined by one-way analysis of variance followed by Tukey's test. Differences between mean values were determined by Student's *t*-test. p values < 0.05 were taken to be statistically significant.

3. Results

3.1. PPARα activation is essential for DHA to inhibit TNFα-induced NFκB

TNFα induces ICAM-1 gene expression by the activation and binding of NFκB to the κB consensus in the ICAM-1 gene promoter (Krunkosky et al., 2000). To investigate whether PPARα plays a role in the suppression of TNFα-induced ICAM-1 expression by DHA in endothelial cells, we first examined the effect of DHA on PPARα activation and TNFα-induced ICAM-1 gene expression. We found that DHA activated PPRE reporter activity similar to the PPARα agonist clofibrate (Fig. 1A). In the presence of TNFα, p65 nuclear translocation was increased, and this increase was suppressed by pretreatment with DHA (Fig. 1B). In contrast, the ability of DHA to inhibit TNFα-induced p65 nuclear translocation was mitigated in the presence of the PPARα antagonist GW6471. EMSA further revealed that TNFα increased NFκB nuclear protein-DNA complex formation, and DHA pretreatment suppressed the TNFα-induced NFκB nuclear protein-DNA binding activity. In the presence of GW6471, this suppression of complex formation by DHA was attenuated (Fig. 1C). Moreover, ICAM-1 promoter activity (Fig. 1D), protein expression (Fig. 1E), and mRNA expression (Fig. 1F) were significantly induced by TNFα ($p < 0.05$), and these enhancements were attenuated by DHA pretreatment. However, the inhibitory effect of DHA on TNFα-induced ICAM-1 promoter activity and gene expression was abolished in the presence of the PPARα antagonist GW6471. These results suggest that PPARα activation is likely to be important for DHA to inhibit TNFα-induced p65 nuclear translocation and NFκB nuclear protein-DNA complex formation.

3.2. Knockdown of PPARα prevents DHA from inhibiting TNFα-induced ICAM-1 expression

To further clarify the actual role of PPARα in the suppression of TNFα-induced ICAM-1 expression by DHA, we used a PPARα knockdown approach. As shown in Fig. 2A, PPARα silencing suppressed the mRNA expression of PPARα, and the ability of DHA to inhibit TNFα-induced ICAM-1 mRNA expression was abrogated. Similar results were found for ICAM-1 protein expression (Fig. 2B). These results suggest that PPARα is necessary for DHA to inhibit TNFα-induced ICAM-1 expression.

3.3. PPARα mediates the inhibition of TNFα-induced monocyte adhesion to the endothelium by DHA

Next, we investigated the role of PPARα in the inhibition by DHA of monocyte attachment to endothelial cells during inflammation. As shown in Fig. 3A, TNFα significantly increased the number of THP-1 cells attached to EA. hy926 cells ($p < 0.05$). With DHA pretreatment, TNFα-induced monocyte adhesion was reversed, whereas this inhibition was abolished in the presence of the PPARα antagonist GW6471. We also studied the role of PPARα expression in TNFα-induced monocyte adhesion. As shown in Fig. 3B, PPARα silencing attenuated the ability of DHA to inhibit TNFα-induced monocyte adhesion, which suggests that the presence of PPARα and subsequent PPARα activation were needed for DHA to inhibit TNFα-induced monocyte adhesion.

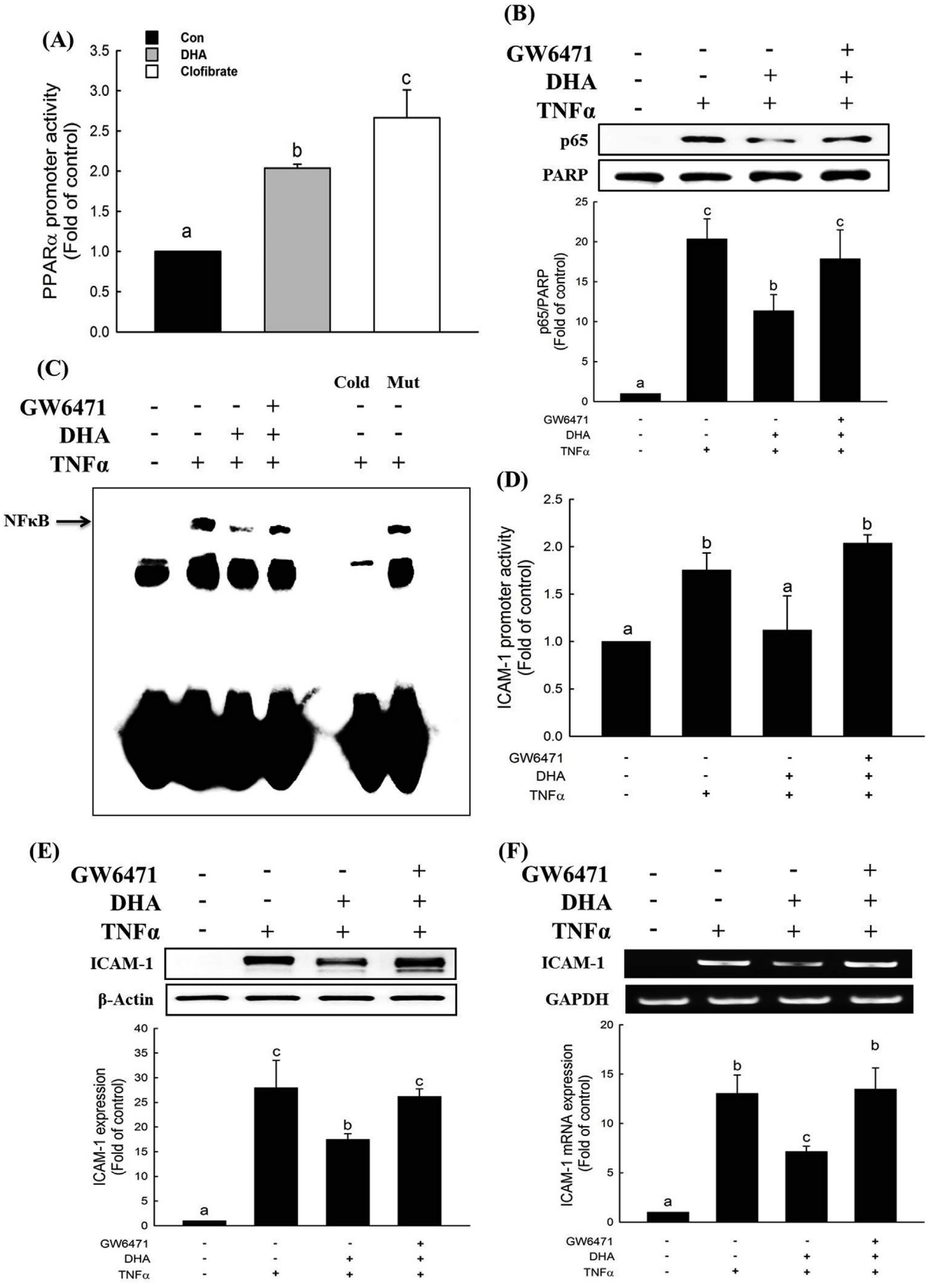
3.4. Effect of autophagy on inhibition of TNFα-induced ICAM-1 expression by DHA

Based on the evidence of previous study, there was a linkage between autophagy blockade and NLRP3 inflammasome activation (Zhou et al., 2011). We were thus interested to know whether autophagy plays a role in the inhibition by DHA of TNFα-induced ICAM-1 expression in vascular endothelial cells. To study this, we transduced EA. hy926 cells with Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B for 16 h and then treated the cells with 100 μM DHA for another 16 h. As shown in Fig. 4A, Q2 represents the percentage of cells with an autophagosome, Q3 represents the percentage of cells with an autolysosome, and Q4 represents the percentage of normal cells. Compared with the control group, DHA and chloroquine diphosphate, an inducer of autophagy, significantly induced autophagosome formation (24.6 ± 3.3% and 45.1 ± 12.9% vs. 7.6 ± 3.3%). Moreover, induction of autophagy by DHA was substantiated via LC3II protein expression. As shown in Fig. 4B, consistent with inhibition of TNFα-induced ICAM-1 protein expression by DHA, the expression of LC3II was significantly increased. Pretreatment with wortmannin (which blocks the formation of autophagosomes) (Lin and Tsai, 2017) abolished the DHA-induced LC3II protein expression and DHA inhibition of TNFα-induced ICAM-1 protein expression. These results suggest that induction of autophagy is involved in the inhibition of TNFα-induced ICAM-1 protein expression by DHA.

4. Discussion

Inflammation is recognized to be a cause of numerous chronic diseases, including CVD, type 2 diabetes, and several neurodegenerative diseases (Tabas and Glass, 2013). Thus, there is increasing interest in remedies that target the inflammatory response. DHA has been shown to possess anti-inflammatory activity (Yang et al., 2013; Maroon and Bost, 2006), and DHA and fish oil are often recommended as adjuvants in the treatment of chronic diseases. In this study, we showed that DHA effectively inhibited TNFα-induced ICAM-1 expression in EA. hy926 cells and that this suppression was likely associated with PPARα, inhibition of NFκB and DNA binding activity, and the induction of autophagy.

It was shown previously that DHA acts as a PPARα/γ ligand and inhibits NFκB binding activity (Adkins and Kelley, 2010). Moreover, DHA was demonstrated to bind with high affinity to PPARα and RXR by use of computational methods (Gani and Sylte, 2008). PPARs are reported to play important roles in anti-inflammation via three distinctive pathways (Desvergne and Wahli, 1999). These are (1) by blockade of membrane receptors mediating the action of inflammatory molecules, (2) by regulating their metabolic fate through suppression of their synthesis or (3) by stimulation of their breakdown. We showed here that DHA significantly activated PPARα ($p < 0.05$) (Fig. 1), which agrees with the findings of Gani and Sylte (2008). NFκB nuclear translocation determines its inflammatory potency by binding to the κB element in the promoter region of target genes. Based on the key role of PPARα in anti-inflammation, we used the ICAM-1 promoter-luciferase construct (pIC339, -339 to 0) (van de Stolpe et al., 1996) to investigate the effect of PPARα on DHA inhibition of TNFα-induced ICAM-1



(caption on next page)

Fig. 1. Activation of PPARα is essential for DHA to inhibit TNFα-induced NFκB. (A) CHO–K1 cells were transiently transfected with a plasmid harboring the PPARα ligand binding domain. After transfection, cells were treated with 100 μM DHA or clofibrate for 24 h followed by detection by the SEAP assay. (B) EA. hy926 endothelial cells were pretreated with 8 μM of the PPARα antagonist GW6471 for 15 min and then with 100 μM DHA for 16 h before being challenged with 1 ng/mL TNFα for an additional 3 h. Aliquots of nuclear extracts (10 μg) were used for Western blot analysis. (C) Aliquots of nuclear extracts (12 μg) were used for EMSA. (D) EA. hy926 endothelial cells transfected with the pIC339 luciferase expression vector were pretreated with 8 μM GW6471 for 15 min and then with 100 μM DHA for 16 h before being challenged with 1 ng/mL TNFα for an additional 6 h. (E) EA. hy926 endothelial cells were pretreated with or without 8 μM GW6471 for 15 min and then with 100 μM DHA for 16 h before being challenged with 1 ng/mL TNFα for an additional 6 h. Aliquots of total protein (10 μg) were used for Western blot analysis. (F) Total RNA was isolated from cells and was subjected to RT-PCR with specific ICAM-1 and GAPDH primers as described in the Materials and methods. Values are means ± SD of three independent experiments. Values not sharing the same letter are significantly different ($p < 0.05$).

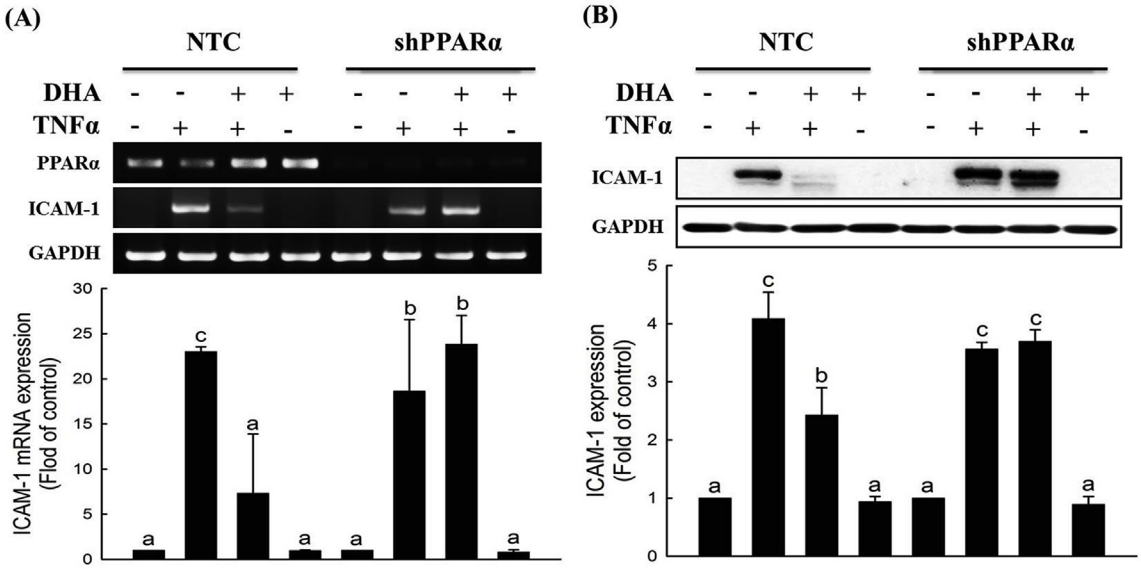


Fig. 2. PPARα expression mediates the inhibition of TNFα-induced ICAM-1 expression by DHA. The shPPARα was used to create a PPARα knockdown model in EA. hy926 cells. Cells were transfected with shPPARα for 24 h and were then treated with 100 μM DHA for 16 h before being challenged with 1 ng/mL TNFα for an additional 6 h. (A) Total RNA was isolated from cells and was subjected to RT-PCR with specific PPARα, ICAM-1, and GAPDH primers as described in the Materials and methods. (B) Aliquots of total protein (10 μg) were used for Western blot analysis. Values are means ± SD of three independent experiments. Values not sharing the same letter are significantly different ($p < 0.05$).

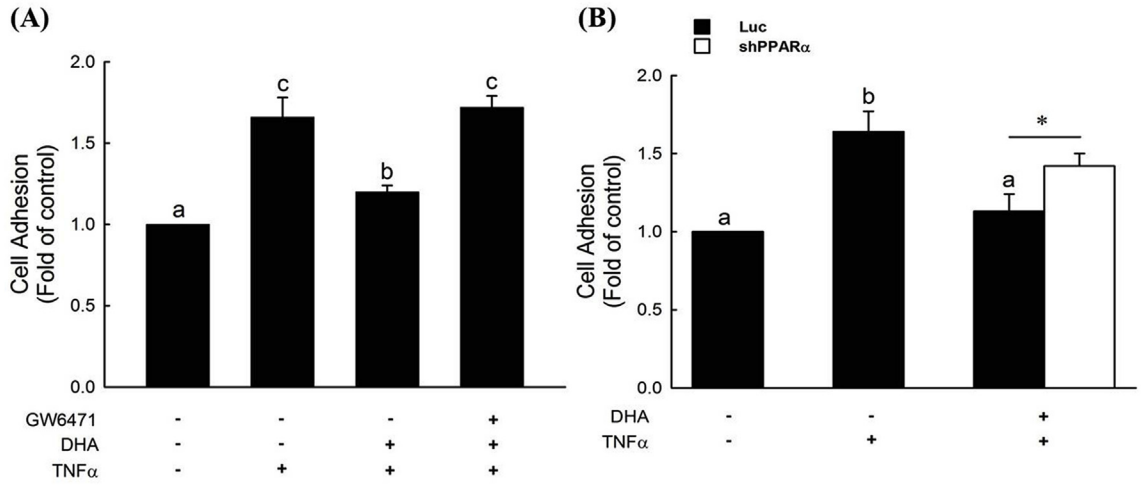


Fig. 3. Both PPARα activation and PPARα expression mediate the inhibition of TNFα-induced THP-1 cell adhesion by DHA. (A) Cells were pretreated with or without 8 μM GW6471 for 15 min and then with 100 μM DHA for 16 h before being challenged with 1 ng/mL TNFα for an additional 6 h. (B) Cells were transfected with shPPARα for 24 h and were then treated with 100 μM DHA for 16 h before being challenged with 1 ng/mL TNFα for an additional 6 h. Values are means ± SD of three independent experiments. *Significantly different from treatment in the shLuc group by Student's *t*-test, $p < 0.05$. Values not sharing the same letter are significantly different ($p < 0.05$).

promoter activity and NFκB activation. As shown in Fig. 1D, DHA significantly inhibited TNFα-induced ICAM-1 promoter activity ($p < 0.05$), whereas this inhibition was dramatically reversed by the PPARα antagonist GW6471 ($p < 0.05$). Moreover, we found a similar effect of GW6471 on the ability of DHA to inhibit TNFα-induced nuclear translocation of p65 NFκB (Fig. 1B) and NFκB and DNA binding activity (Fig. 1C). These results suggest that PPARα plays a critical role in DHA fighting against TNFα-induced ICAM-1 promoter activity and p65 NFκB nuclear translocation as well as NFκB and DNA binding activity. The role of PPARα is likely associated with blockade of the TNFα

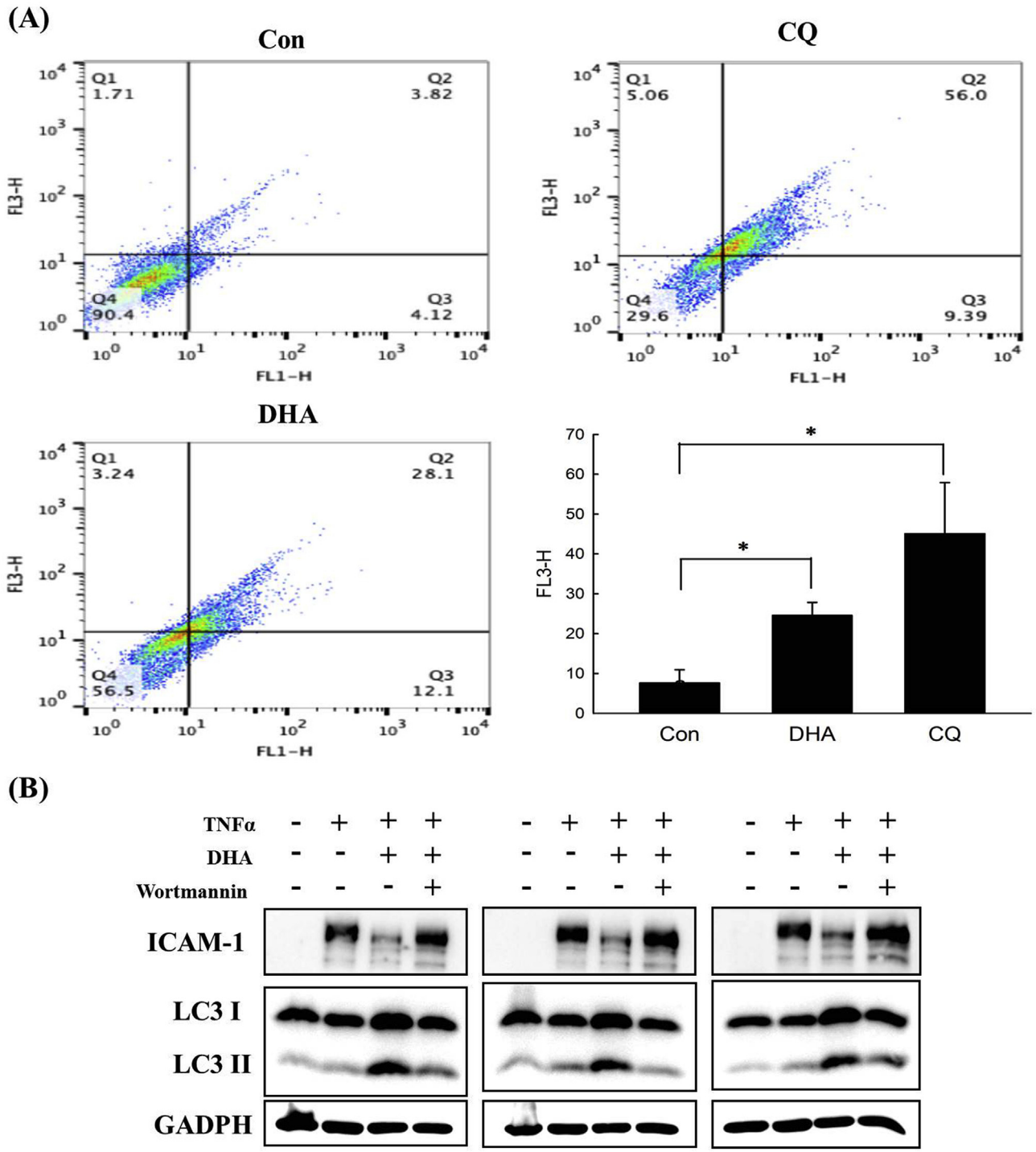


Fig. 4. Activation of autophagy mediates the inhibition of TNFα-induced ICAM-1 expression by DHA. (A) DHA-induced autophagy. Cells were treated with 100 μM DHA for 16 h. Chloroquine diphosphate (CQ) was added to cells and the cells were incubated for 1 h as a positive control. *Significantly different from control group by Student's *t*-test, $p < 0.01$. (B) Cells were pretreated with 0.5 μM wortmannin for 1 h and were then treated with 100 μM DHA for 16 h before being challenged with 1 ng/mL TNFα for an additional 6 h. Aliquots of total protein (10 μg) were used for Western blot analysis.

receptor and/or stimulation of TNFα breakdown as proposed by Desvergne and Wahli (1999); however, a definite answer warrants further study. Furthermore, we investigated the essentiality of PPARα in the inhibition of TNFα-induced ICAM-1 expression. As shown in Fig. 1E and F, DHA significantly inhibited TNFα-induced ICAM-1 protein and mRNA expression ($p < 0.05$), whereas this inhibition was overwhelmed by GW6471. To verify the importance of PPARα in the anti-inflammatory effect of DHA, a PPARα knockdown model was created. As shown in Fig. 2A and B, PPARα silencing abrogated the inhibition of TNFα-induced ICAM-1 mRNA and protein expression by DHA. These results suggest that PPARα is essential for DHA to suppress TNFα-induced ICAM-1 mRNA and protein expression. Also, the results support the findings of Okamoto et al. who showed that the PPARα agonist fenofibrate inhibited IL-1β-induced nuclear translocation of p65 NFκB and inflammatory gene expression (Okamoto et al., 2005).

The recruitment of leukocytes from the bloodstream to the site of inflammation is a hallmark of acute inflammation (Hossain et al., 2013). Proinflammatory cytokines such as IL-1β, IL-6, IL-8, and TNFα

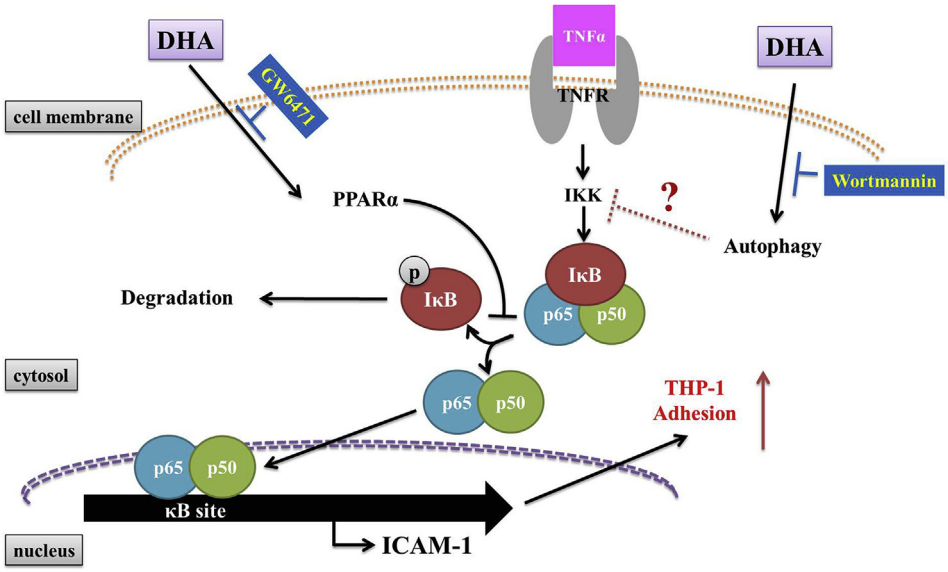


Fig. 5. Scheme summarizing the inhibition of TNFα-induced vascular inflammation by DHA: PPARα is activated, p65 nuclear translocation is inhibited, NFκB and DNA binding activity are inhibited, ICAM-1 promoter activity is inhibited, ICAM-1 expression is inhibited, and autophagy is activated. As a result, THP-1 cell adhesion to TNFα-stimulated EA. hy926 cells is attenuated.

(Oprée and Kress, 2000) up-regulate the expression of a number of endothelial adhesion molecules, including ICAM-1, ICAM-2, vascular cell adhesion molecule 1 (VCAM-1), E-selectin and P-selectin, PECAM-1, and MAdCAM-1 (Golias et al., 2007). ICAM-1 mediates leukocyte adhesion on endothelial cells before their transmigration via an interaction with the β2 integrins expressed on leukocytes (Williams et al., 2011). DHA has been shown to inhibit TNFα-induced ICAM-1 expression and monocyte adhesion (Yang et al., 2013). In the present study, we further demonstrated the key role of PPARα in the inhibition by DHA of TNFα-induced THP-1 adhesion. As shown in Fig. 3A, DHA significantly suppressed THP-1 adhesion to TNFα-treated endothelial cells ($p < 0.05$), and this result was in line with our previous study, which proposed that the inhibitory effect of DHA was likely associated with the induction of HO-1 expression (Yang et al., 2013). As shown in Fig. 3B, the inhibition of TNFα-induced monocyte adhesion by DHA was reversed by shPPARα, which suggests the involvement of PPARα in the inhibition by DHA of TNFα-induced THP-1 adhesion.

Autophagy is a normal physiologic process in the body (Mao et al., 2016) and results in the maintenance of cellular homeostasis. Accumulating evidence has shown a linkage between dysregulated autophagy and the pathogenesis of several human inflammatory diseases. Meissner et al. (2010) have shown that suppression of autophagy enhances the cytoplasmic accumulation of mutant SOD1, activates microglial inflammasomes, markedly increases the release of IL-1β, and accelerates the progression of amyotrophic lateral sclerosis (ALS). Beclin-1 protein is a key molecule for autophagosome formation (Sinha and Levine, 2008). Decreased levels of beclin-1 lead to defective autophagy, which drives lung inflammation in cystic fibrosis (CF) (Luciani et al., 2010). Luciani et al. (2010) suggest that the restoration of beclin-1 and autophagy may be a novel approach for CF remedy. In our previous study, we showed that DHA has an anti-inflammatory effect on TNFα-induced ICAM-1 expression in EA. hy926 cells (Yang et al., 2013). Due to emerging evidence of a close relationship between autophagy and anti-inflammation, we decided to explore the role of autophagy in DHA's inhibition of TNFα-induced ICAM-1 expression. As shown in Fig. 4A, DHA significantly induced autophagy as evidenced by an increase in the proportion of cells with autophagosomes. Moreover, the role of autophagy in the inhibition of TNFα-induced ICAM-1 expression by DHA was reinforced by use of wortmannin (which blocks the formation of the autophagosome). Pretreatment with wortmannin abolished not only DHA-induced LC3II protein expression but also DHA inhibition of TNFα-induced ICAM-1 expression (Fig. 4B). These results were consistent with the findings of Huang et al. who showed that resveratrol suppresses TNFα-induced ICAM-1 mRNA expression via autophagy by using siAtg16L1 (Huang et al., 2017).

5. Conclusions

The findings of this study are presented schematically in Fig. 5. The novelty of this study is the discovery that PPARα and autophagy induction are involved in the anti-inflammatory effect of DHA on the endothelium. The anti-inflammatory activity of DHA in the vascular endothelium partially explains its cardioprotective effect.

Funding

This study was supported by grants CMU105-ASIA-13 and CMU107-ASIA-04 from China Medical University and Asia University, Taiwan.

Author contributions

The author's responsibilities were as follows: Chen H–W and Lii C–K designed the study; Lin H–C, Lin A-H, Li C–C, Tsai C–H, Pan S–K, Yang Y–C, Hua ng C–S and Reshi R conducted the research; and Lii C–K analyzed the data. Chen H–W wrote the manuscript. Chen H–W has the primary responsibility for the final content. All of the authors have read and approved the final manuscript.

Financial disclosure

The authors have nothing to disclose.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The human endothelial cell line EA. hy926 was a kind gift from Dr. T. S. Wang, Chung Shan Medical University, Taichung, Taiwan. Chinese hamster ovary cell line CHO–K1 was a kind gift from Dr. C. C. Li, Chung Shan Medical University, Taichung, Taiwan.

References

Adkins, Y., Kelley, D.S., 2010. Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. J. Nutr. Biochem. 21, 781–792.

Cheng, W.L., Lii, C.K., Chen, H.W., Lin, T.H., et al., 2004. Contribution of conjugated linoleic acid to the suppression of inflammatory responses through the regulation of the NF-kappaB pathway. J. Agric. Food Chem. 52, 71–78.

Desvergne, B., Wahli, W., 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr. Rev. 20, 649–688.

Gani, O.A., Sylte, I., 2008. Molecular recognition of docosahexaenoic acid by peroxisome proliferator-activated receptors and retinoid-X receptor alpha. J. Mol. Graph. Model. 27, 217–224.

Golias, C., Tsoutsi, E., Matziridis, A., Makridis, P., et al., 2007. Review. Leukocyte and endothelial cell adhesion molecules in inflammation focusing on inflammatory heart disease. In Vivo 21, 757–769.

He, C., Klionsky, D.J., 2009. Regulation mechanisms and signaling pathways of autophagy. Annu. Rev. Genet. 43, 67–93.

Heneka, M.T., Landreth, G.E., 2007. PPARs in the brain. Biochim. Biophys. Acta 1771, 1031–1045.

Hossain, M., Qadri, S.M., Su, Y., Liu, L., 2013. ICAM-1-mediated leukocyte adhesion is critical for the activation of endothelial LSP1. Am. J. Physiol. Cell Physiol. 304, 895–904.

Hoyert, D.L., 2012. 75 years of mortality in the United States, 1935-2010. NCHS data brief 88, 1–8.

Huang, F.C., Kuo, H.C., Huang, Y.H., Yu, H.R., et al., 2017. Anti-inflammatory effect of resveratrol in human coronary arterial endothelial cells via inductionof autophagy: implication for the treatment of Kawasaki disease. BMC Pharmacol. Toxicol. 18, 3.

Inoue, I., Goto, S., Matsunaga, T., Nakajima, T., et al., 2001. The ligands/activators for peroxisome proliferator-activated receptor alpha (PPARalpha) and PPARgamma increase Cu^{2+}, Zn^{2+}-superoxide dismutase and decrease p22phox message expressions in primary endothelial cells. Metabolism 50, 3–11.

Jing, K., Song, K.S., Shin, S., Kim, N., et al., 2011. Docosahexaenoic acid induces autophagy through p53/AMPK/mTOR signaling and promotes apoptosis in human cancer cells harboring wild-type p53. Autophagy 7, 1348–1358.

Jump, D.B., Depner, C.M., Tripathy, S., 2012. Omega-3 fatty acid supplementation and cardiovascular disease. J. Lipid Res. 53, 2525–2545.

Karin, M., Delhase, M., 2000. The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin. Immunol. 12, 85–98.

Krunkosky, T.M., Fischer, B.M., Martin, L.D., Jones, N., et al., 2000. Effects of TNF-alpha on expression of ICAM-1 in human airway epithelial cells in vitro. Signaling pathways controlling surface and gene expression. Am. J. Respir. Cell Mol. Biol. 22, 685–692.

Lefebvre, P., Chinetti, G., Fruchart, J.C., Staels, B., 2006. Sorting out the roles PPAR alpha in energy metabolism and vascular homeostasis. J. Clin. Investig. 116, 571–580.

Lei, Y.P., Chen, H.W., Sheen, L.Y., Lii, C.K., 2008. Diallyl disulfide and diallyl trisulfide suppress oxidized LDL-induced vascular cell adhesion molecule and E-selectin expression through protein kinase A- and protein kinase B-dependent signaling pathways. J. Nutr. 138, 996–1003.

Levine, B., Yuan, J., 2005. Autophagy in cell death: an innocent convict? J. Clin. Investig. 115, 2679–2688.

Libby, P., 2002. Inflammation in atherosclerosis. Nature 420, 868–874.

Lin, C.Y., Tsai, C.W., 2017. Carnosic acid attenuates 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y cells by inducing autophagy through an enhanced interaction of parkin and beclin 1. Mol. Neurobiol. 54, 2813–2822.

Liu, K.L., Yang, Y.C., Yao, H.T., Chia, T.W., et al., 2016. 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. Mol. Nutr. Food Res. 60, 430–443.

Luciani, A., Villella, V.R., Esposito, S., Brunetti-Pierri, N., et al., 2010. Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. Nat. Cell Biol. 12, 863–875.

Lusis, A.J., 2000. Atherosclerosis. Nature 407, 233–241.

Mao, Y., Yu, F., Wang, J., Guo, C., et al., 2016. Autophagy: a new target for nonalcoholic fatty liver disease therapy. Hepat. Med. 8, 27–37.

Maroon, J.C., Bost, J.W., 2006. Omega-3 fatty acids (fish oil) as an anti-inflammatory: an alternative to nonsteroidal anti-inflammatory drugs for discogenic pain. Surg. Neurol. 65, 326–331.

Meissner, F., Molawi, K., Zychlinsky, A., 2010. Mutant superoxide dismutase 1-induced IL-1 beta accelerates ALS pathogenesis. Proc. Natl. Acad. Sci. U.S.A. 107, 13046–13050.

Oh, J.H., Park, E.J., Park, J.W., Lee, J., et al., 2010. A novel cyclin-dependent kinase inhibitor down-regulates tumor necrosis factor-alpha (TNF-alpha)-induced expression of cell adhesion molecules by inhibition of NF-kappaB activation in human pulmonary epithelial cells. Int. Immunopharmacol. 10, 572–579.

Okamoto, H., Iwamoto, T., Kotake, S., Momohara, S., et al., 2005. Inhibition of NF-kappaB signaling by fenofibrate, a peroxisome proliferator-activated receptor-alpha ligand, presents a therapeutic strategy for rheumatoid arthritis. Clin. Exp. Rheumatol. 23, 323–330.

Oprée, A., Kress, M., 2000. Involvement of the proinflammatory cytokines tumor necrosis factor-alpha, IL-1 beta, and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related peptide release from rat skin. J. Neurosci. 20, 6289–6293.

Penumetcha, M., Santanam, N., 2012. Nutraceuticals as ligands of PPARγ. PPAR Res. 2012, 858352.

Rigamonti, E., Chinetti-Gbaguidi, G., Staels, B., 2008. Regulation of macrophage functions by PPAR-alpha, PPAR-gamma, and LXRs in mice and men. Arterioscler. Thromb. Vasc. Biol. 28, 1050–1059.

Sinha, S., Levine, B., 2008. The autophagy effector Beclin 1: a novel BH3-only protein. Oncogene 27 (Suppl. 1), S137–S148.

Tabas, I., Glass, C.K., 2013. Anti-inflammatory therapy in chronic disease: challenges and opportunities. Science 339, 166–172.

Taiwan Health and Welfare Report 2017.

Tang, S.C., Wu, M.F., Wong, R.H., Liu, Y.F., et al., 2011. Epigenetic mechanisms for silencing glutathione S-transferase m2 expression by hypermethylated specificity protein 1 binding in lung cancer. Cancer 117, 3209–3221.

van de Stolpe, A., van der Saag, P.T., 1996. Intercellular adhesion molecule-1. J. Mol. Med. 74, 13–33.

Wan, Y.J., Cai, Y., Lungo, W., Fu, P., et al., 2000. Peroxisome proliferator-activated receptor alpha-mediated pathways are altered in hepatocyte-specific retinoid X receptor alphadeficient mice. J. Biol. Chem. 275, 28285–28290.

Williams, M.R., Azcutia, V., Newton, G., Alcaide, P., et al., 2011. Emerging mechanisms of neutrophil recruitment across endothelium. Trends Immunol. 32, 461–469.

Yang, Y.C., Lii, C.K., Wei, Y.L., Li, C.C., et al., 2013. Docosahexaenoic acid inhibition of inflammation is partially via cross-talk between Nrf2/heme oxygenase 1 and IKK/NF-κB pathways. J. Nutr. Biochem. 24, 204–212.

Zhou, R., Yazdi, A.S., Menu, P., Tschopp, J., 2011. A role for mitochondrial in NLRP3 inflammasome activation. Nature 469, 221–225.