Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 24 (2013) 204–212

Journal of Nutritional Biochemistry

Docosahexaenoic acid inhibition of inflammation is partially via cross-talk between Nrf2/heme oxygenase 1 and IKK/NF-κB pathways☆,☆☆

Ya-Chen Yang[a], Chong-Kuei Lii[b,1], Yu-Ling Wei[b], Chien-Chun Li[c], Chia-Yang Lu[c], Kai-Li Liu[c], Haw-Wen Chen[b,*]

[a]Department of Health and Nutrition Biotechnology, Asia University, Taichung 413, Taiwan
[b]Department of Nutrition, China Medical University, Taichung 404, Taiwan
[c]Department of Nutrition, Chung Shan Medical University, Taichung 402, Taiwan

Received 15 January 2012; received in revised form 26 April 2012; accepted 1 May 2012

Abstract

We examined the underlying mechanisms involved in n-3 docosahexaenoic acid (DHA) inhibition of inflammation in EA.hy926 cells. The present results demonstrated that pretreatment with DHA (50 and 100 μM) inhibited tumor necrosis factor-alpha (TNF-α)-induced intercellular adhesion molecule 1 (ICAM-1) protein, mRNA expression and promoter activity. In addition, TNF-α-stimulated inhibitory kappa B (IκB) kinase (IKK) phosphorylation, IκB phosphorylation and degradation, p65 nuclear translocation, and nuclear factor-κB (NF-κB) and DNA binding activity were attenuated by pretreatment with DHA. DHA triggered early-stage and transient reactive oxygen species (ROS) generation and significantly increased the protein expression of heme oxygenase 1 (HO-1), induced nuclear factor erythroid 2-related factor 2 (Nrf2) translocation to the nucleus and up-regulated antioxidant response element (ARE)-luciferase reporter activity. Moreover, DHA inhibited Nrf2 ubiquitination and proteasome activity. DHA activated Akt, p38 and ERK1/2 phosphorylation, and specific inhibitors of respective pathways attenuated DHA-induced Nrf2 nuclear translocation and HO-1 expression. Transfection with HO-1 siRNA knocked down HO-1 expression and partially reversed the DHA-mediated inhibition of TNF-α-induced p65 nuclear translocation and ICAM-1 expression. Importantly, we show for the first time that HO-1 plays a down-regulatory role in NF-κB nuclear translocation, and inhibition of Nrf2 ubiquitination and proteasome activity are involved in increased cellular Nrf2 level by DHA. In this study, we show that HO-1 plays a down-regulatory role in NF-κB nuclear translocation and that the protective effect of DHA against inflammation is partially via up-regulation of Nrf2-mediated HO-1 expression and inhibition of IKK/NF-κB signaling pathway.
© 2013 Elsevier Inc. All rights reserved.

Keywords: Docosahexaenoic acid (DHA); Heme oxygenase 1 (HO-1); Intercellular adhesion molecule 1 (ICAM-1); Nuclear factor erythroid 2-related factor 2 (Nrf2); Tumor necrosis factor-alpha (TNF-α)

1. Introduction

Inflammation is recognized as a major contributing factor to many cardiovascular events [1]. Atherosclerosis, a chronic inflammatory disease of the vasculature, is characterized by infiltration of leucocytes, deposition of lipids and thickening of the vascular wall in response to cytokines [2]. Leukocyte recruitment is a multistep process that is predominantly mediated by cellular adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1) and selectins. Studies have shown that tumor necrosis factor-alpha (TNF-α), the proinflammatory cytokine, is commonly found in atherosclerotic lesions and can induce the expression of ICAM-1 and VCAM-1, which are critically dependent on the activation of nuclear factor-κB (NF-κB) [3]. In quiescent cells, NF-κB is sequestered in the cytoplasm through its interaction with the inhibitory kappa B (IκB) family. In response to stimulation, IκBα is phosphorylated by the IκB kinase (IKK) complex and subsequently degraded [4]. IκB degradation allows NF-κB translocation to the nucleus, where it can bind to the κB element of the promoter of target genes [4].

Heme oxygenase 1 (HO-1) is an inducible enzyme responsible for the rate-limiting step in heme degradation [5]. HO-1 can be induced by a variety of stress-related cellular stimuli [5,6]. HO-1 expression plays a role in several pathological states, such as atherosclerosis and inflammation, wherein it confers cytoprotection [6,7]. HO-1 induction reduces atherosclerotic lesion size in Watanabe heritable hyperlipidemic rabbits [8] and in low-density lipoprotein (LDL)-receptor knockout mice [9]. Moreover, transgenic mice deficient in HO-1 of an apolipoprotein E null background exhibit accelerated and more advanced atherosclerotic lesion formation in response to a Western diet [10]. HO-1 expression is primarily regulated at the transcriptional level, and its induction is linked to the transcription factor nuclear

☆ This study was supported by grants NSC 101-2313-B-039-007-MY3 and CMU100-ASIA-09.
☆☆ Author disclosures: No conflicts of interest are declared.
* Corresponding author. Tel.: +886 4 22053366x7520; fax: +886 4 2206 2891.
E-mail address: chenhw@mail.cmu.tw (H.-W. Chen).
[1] Chong-Kuei Lii contributed equally to the first author in this work.

0955-2863/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jnutbio.2012.05.003

factor erythroid 2-related factor 2 (Nrf2) [11]. Under basal conditions, Nrf2 is sequestered in the cytoplasm by binding to Kelch-like ECH-associated protein 1 (Keap1) [12]. When disrupted by electrophilic antioxidants, Nrf2 is released from Keap1 and translocates to the nucleus, dimerizes with Maf and activates the transcription of genes containing the antioxidant response element (ARE) in the promoter region [12].

The long-chain n-3 polyunsaturated fatty acids (PUFAs) in fish oil, especially eicosapentaenoic acid and docosahexaenoic acid (DHA), have well-known anti-inflammatory [13], immunoregulatory [14] and antitumor properties [15]. Dietary intake of n-3 PUFAs is associated with a reduced risk of atherosclerosis [16], and this is considered to play a pivotal role in the prevention of cardiovascular disease. Epidemiological evidence indicates that supplementation with n-3 PUFAs regulates inflammation partially by improving endothelial function [17]. DHA significantly decreases cytokine-induced adhesion molecule expression [18], diminishes the adhesion of leukocytes to activated endothelial cells [19] and inhibits the production of cytokines by endothelial cells [20].

Although the anti-inflammatory effect of DHA has been studied previously, in addition to NF-κB pathway, the molecular mechanism underlying the DHA-mediated inhibition of TNF-α-induced ICAM-1 expression in human vascular endothelial cells remains unclear. The aim of this study was to investigate DHA inhibition of TNF-α-induced inflammation as well as the possible molecular mechanism(s) involved.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle medium (DMEM), RPMI 1640, RPMI-1640 (without phenol red), OPTI-MEM and penicillin/streptomycin were from GIBCO/BRL (Grand Island, NY, USA); fetal bovine serum (FBS) was from HyClone (Logan, UT, USA); DHA was from Cayman Chemical (Ann Arbor, MI, USA); human TNF-α was from Sigma-Aldrich (St. Louis, MO, USA); Z-Leu-Leu-Leu-CHO (MG-132) was from Boston Biochem (Cambridge, MA, USA); 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM) was from Molecular Probes (Eugene, OR, USA); 2,7-dichlorofluorescin diacetate (H_2DCFDA) and TRIzol reagent were from Invitrogen (Carlsbad, CA, USA); antibody against HO-1 and fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) was from Calbiochem (Darmstadt, Germany); antibodies against Nrf2, IκBα, IKKα/IKKβ, JNK, phospho-JNK, ERK and p38 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against ICAM-1, phospho-IκBα (Ser32/36), phospho-IKKα (Ser180)/IKKβ (Ser181), PARP, phospho-ERK and phospho-p38 were from Cell Signaling Technology (Boston, MA, USA); and antibody against p65 was from BD Bioscience (San Jose, CA, USA).

2.2. Fatty acid preparation

DHA-fatty acid-free bovine serum albumin solutions were prepared as described by Spector [21]. Sodium salt of DHA was prepared and complexed with fatty acid-free bovine serum albumin at a 6:1 molar ratio before addition to the culture medium. At the same time, 0.1% butylated hydroxytoluene and 20 μmol/L α-tocopheryl succinate were added to the culture medium to prevent lipid peroxidation.

2.3. Cell cultures

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 $NaHCO_3$, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator as described by Liao et al. [22]. 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 at 37°C in a 5% CO_2 humidified incubator.

2.4. Cell viability assay

EA.hy926 cells were grown to 70%–80% confluence and were then treated with different concentrations of DHA for 24 h followed by incubation with 1 ng/ml TNF-α for an additional 6 h. Afterward, cell viability assay was performed according to our previous study [23].

2.5. Western blotting analysis and electrophoretic mobility shift assay (EMSA)

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, 0.1% 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 at 14,000g for 20 min at 4°C. The resulting supernatant was used as a cellular protein. Nuclear protein preparation was performed as described previously [24]. The total protein was analyzed by use of the Coomassie Plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL, USA). For Western blotting, equal amounts of cellular and nuclear proteins were electrophoresed in a sodium dodecyl sulfate (SDS)–polyacrylamide gel, and proteins were then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). 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. The membranes were probed with antibodies. The membranes were then probed with the secondary antibody labeled with horseradish peroxidase. 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). EMSA was performed according to our previous study [24]. A total of 4 μg nuclear proteins was used for the assay.

2.6. RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from EA.hy926 cells using TRIzol reagent and frozen at −70°C until RT-PCR analysis was performed. We used 0.2 μg of total RNA 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 was conducted in a thermocycler 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′-TGAAGGCCACCCCAGAGGACAAC-3′; reverse, 5′-CCCATTATGACTGCGGCTGCTGCTACC-3′), HO-1 (forward, 5′-CTGAGTTCATGAGGAACTTTCAGAAG-3′; reverse, 5′-TGGTACAGGGAGGCCATCAC-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5′-CCATCACCATCTTCCAGGAG-3′; reverse, 5′-CCTGCTTCACCACCTTCTTG-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 HO-1 was achieved when samples were heated to 95°C for 5 min and then immediately cycled 39 times through a 1-min denaturing step at 95°C, a 1-min annealing step at 55°C and a 2-min elongation step at 72°C. The GAPDH cDNA level was used as the internal standard. PCR products were resolved in a 1% agarose gel and were scanned by a Digital Image Analyzer (Alpha Innotech) and quantitated with ImageGauge software.

2.7. Plasmids, transfection and luciferase assay

A 2× ARE fragment containing tandem repeats of double-stranded oligonucleotides spanning the Nrf2 binding site, 5′-TGACTCAGCA-3′, was introduced into the pGL3 promoter-luciferase plasmid. 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 [25]. All subsequent transfection experiments were performed by using Nanofectin reagent (PAA, Pasching, Austria) according to the manufacturer's instructions. Cells were transiently transfected with 0.4 μg of pIC339 or pGL3 plasmid and 0.2 μg of β-galactosidase plasmid by using 1 μl of Nanofectin in OPTI-MEM medium for 8 h. After transfection, cells were changed to DMEM medium and treated with DHA for 16 h before being challenged with TNF-α for an additional 6 h. Cells were then washed twice with cold PBS, scraped with lysis buffer and centrifuged at 14,000g for 3 min. The supernatant was collected for the measurement of luciferase and β-galactosidase activities. The luciferase activity was measured by using a Luciferase Assay Kit (Promega, Madison, WI, USA) in a microplate luminometer (TROPIX TR-717, Applied Biosystems). 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. RNA interference by small interfering RNA (siRNA) of HO-1

Predesigned siRNAs against human HO-1 and nontargeting control-pool siRNA were purchased from Dharmacon Inc. (Lafayette, CO, USA). The four siRNAs against the human HO-1 gene are (a) AUGCUGAGUUCAUGAGGAA, (b) ACACUCAGCUUUCUGGUGG, (c) CAGUUGCUGGUAGGGCUUUA and (d) AGAUUGAGCGCAACAAGGA. Cells were transfected with HO-1 siRNA SMARTpool by using DharmaFECT1 transfection reagent (Thermo) according to the manufacturer's instructions. Nontargeting siRNA construct (NC) was used as negative control. Specific silencing was confirmed by at least three independent Western blotting assays with cellular extracts 8 h after transfection.

2.9. Immunoprecipitation

Total cell lysates were diluted to 2 μg/μl with IP buffer, incubated with 0.6 μg anti-Nrf2 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. IP buffer was as follows: 40 mM Tris–HCl (pH 7.5), 1% NP-40, 150 mM EGTA, 1 mM DTT, 1 mM PMSF, 20 mM NaF, 1 μg/μl aprotinin, 1 μg/μl leupeptin and 1 mM sodium vanadate. 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.

2.10. Measurement of proteasome activity

The chymotrypsin-like activity of the proteasome was assessed in cell lysates by using the synthetic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (Calbiochem) as described previously [26].

2.11. Reactive oxygen species (ROS) measurement

Detection of intracellular oxidative states was performed by using the probe H_2DCFDA [27]. Briefly, cells were grown to 60%–70% confluence and then serum-starved in DMEM supplemented with 0.5% (v/v) FBS for an additional 2 days. The cells were then stabilized in serum-free DMEM without phenol red for at least 30 min before exposure to DHA for the indicated time periods. Cells were then incubated for 10 min with the ROS-sensitive fluorophore H_2DCFDA (10 μM). 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.

2.12. Monocyte adhesion assay

EA.hy926 cells in 12-well plates were allowed to grow to 80% confluence and were then pretreated with 50 or 100 μM DHA for 16 h followed by incubation with 1 ng/ml TNF-α for an additional 6 h. Afterward, monocyte adhesion assay was performed according to our previous study [23].

2.13. Statistical analysis

Data were analyzed by using analysis of variance (SAS Institute, Cary, NC, USA). The significance of the difference among mean values was determined by one-way analysis of variance followed by Tukey's test, and the difference between mean values was determined by Student's *t* test. *P* values<.05 were taken to be statistically significant.

3. Results

3.1. Effect of DHA on cell viability in the presence or absence of TNF-α

As measured by the MTT assay, the cell viabilities of EA.hy926 cells treated with TNF-α alone; 50, 100 or 200 μM DHA; or TNF-α and 50, 100 or 200 μM DHA were 99.7%±2.7%, 103.4%±2.2%, 93.6%±5.8%, 61.3%±7.6%, 100%±6.6%, 90.4%±4.3% and 61.1%±4.4%, respectively, compared with the unstimulated controls (100%). Thus, there were no adverse effects on the growth of cells up to a concentration of 100 μM DHA in the presence or absence of 1 ng/ml TNF-α, which was used

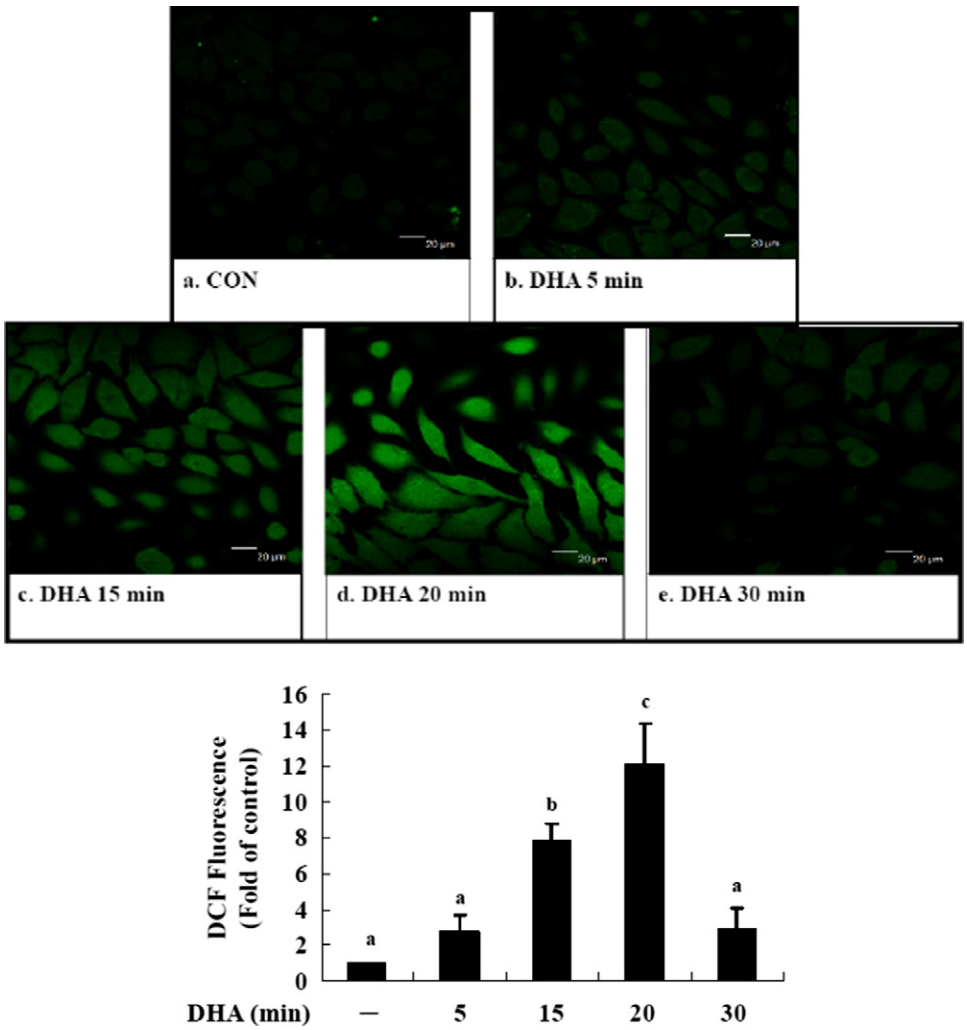

Fig. 1. Effect of DHA on ROS generation. Cells were treated with 100 μM DHA for the indicated time periods and followed by incubation with 10 μM ROS-sensitive fluorophore H_2DCF-DA. Quantitative induction is shown as an increase in the treated cells relative to the control. Values are means±S.D. of three independent experiments. Values not sharing the same letter are significantly different (P <.05).

to induce the expression of ICAM-1. The highest concentration of DHA used in the present study was 100 μM.

3.2. DHA inhibits TNF-α-induced ICAM-1 expression, promoter activity and HL-60 cell adhesion

ICAM-1, a biomarker of inflammation, was shown to be induced by proinflammatory cytokine TNF-α in a previous study [23]. The anti-inflammatory effect of DHA was evaluated by measuring its inhibition of TNF-α-induced ICAM-1 expression. The protein expression of ICAM-1 was significantly suppressed by DHA pretreatment in a dose- and time-dependent manner (Supplemental Fig. 1A). In parallel, DHA also inhibited TNF-α-induced ICAM-1 mRNA expression (Supplemental Fig. 1B). In promoter activity assays performed with an ICAM-1 promoter-luciferase reporter plasmid, TNF-α-induced ICAM-1 promoter activity was inhibited by 50 and 100 μM DHA (Supplemental Fig. 1C). Also, DHA decreased the adhesion of HL-60 cells to TNF-α-stimulated endothelial cells (Supplemental Fig. 1D). Thus, DHA was shown to have anti-inflammatory effect by inhibiting ICAM-1 expression and monocyte adhesion.

3.3. DHA inhibits TNF-α-induced NF-κB signaling pathway

The human proximal ICAM-1 promoter–enhancer region (−346 to −24) contains several putative recognition sequences for a variety of transcriptional activators [25]. Among these, NF-κB binding to the κB binding site in the promoter plays an important role in TNF-α-induced ICAM-1 expression by activating IKKα/β. Therefore, we next determined whether NF-κB activation was inhibited by DHA. As shown in Supplemental Fig. 2A, TNF-α induced both IKKα and IKKβ phosphorylation, and the activation of IKKα and IKKβ was significantly attenuated by DHA pretreatment. In addition, TNF-α caused the phosphorylation and subsequent degradation of IκBα (Supplemental Fig. 2B). However, the phosphorylation of IκBα induced by TNF-α was attenuated by pretreatment with DHA at 5 min, and the degradation was decreased by pretreatment with DHA or MG132 (a proteasome

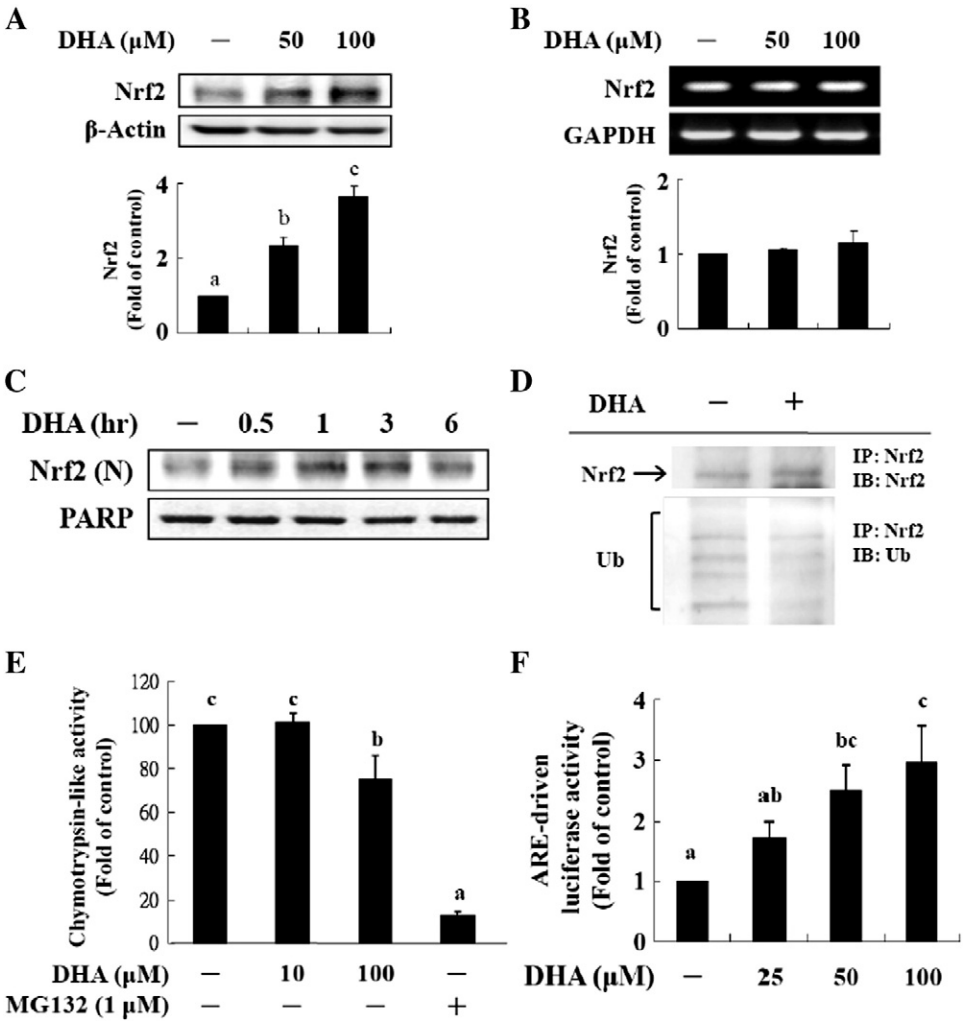

Fig. 2. Effect of DHA on Nrf2/ARE signaling pathway. (A) Cells were treated with 50 and 100 μM DHA for 8 h. Aliquots of total protein (20 μg) were used for Western blot analysis. (B) Total RNA was isolated from cells and was subjected to RT-PCR. (C) Nuclear extract [N] from cells was prepared after treatment with 100 μM DHA for the indicated time periods. (D) Immunoprecipitation of Nrf2 in the cell lysates of DHA-treated cells. Western blot analysis was used to detect Nrf2 and ubiquitinated-Nrf2, which were pulled down by anti-Nrf2 antibody. (E) Cells were pretreated with DHA or 1 μM MG132 for 16 h. Chymotrypsin-like proteasome activity was measured. (F) Cells were transfected with the ARE-luciferase construct (ARE) for 8 h and were then stimulated with 25–100 μM DHA for an additional 16 h. The cells were then lysed and analyzed for luciferase activity. Values are means±S.D. of three independent experiments. Values not sharing the same letter are significantly different ($P < .05$).

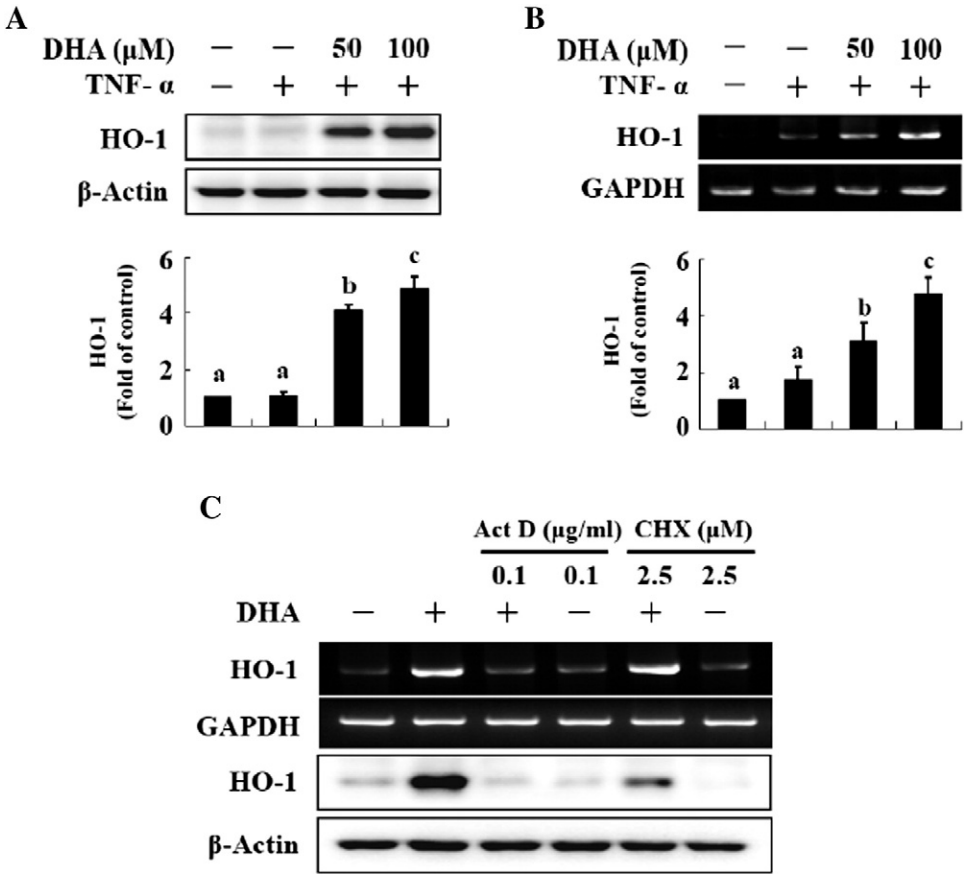

Fig. 3. DHA induces HO-1 expression in EA.hy926 cells. (A) Cells were pretreated with 50 and 100 μM DHA for 8 h followed by incubation with 1 ng/ml TNF-α for another 6 h. Aliquots of total protein (20 μg) were used for Western blot analysis. (B) Total RNA was isolated from cells and was subjected to RT-PCR. (C) Cells were pretreated with either 0.1 μg/ml Act D or 5 μM CHX for 4 h followed by incubation with 50 μM DHA for an additional 16 h. Values are means±S.D. of three independent experiments. Values not sharing the same letter are significantly different ($P < .05$).

inhibitor) (Supplemental Fig. 2B). MG132 is a chymotrypsin-like protease inhibitor [28]. Next, we demonstrated that the nuclear p65 content was increased by TNF-α, and this effect was time-dependently attenuated by pretreatment with DHA (Supplemental Fig. 2C). A significant effect was observed for pretreatment for 8 h and longer. EMSA further revealed that TNF-α increased NF-κB nuclear protein–DNA complex formation and that pretreatment with DHA resulted in the inhibition of NF-κB nuclear protein–DNA binding activity (Supplemental Fig. 2D). These results suggest that DHA effectively inhibits activated IKK/NF-κB signaling pathway.

3.4. DHA triggers early-stage and transient ROS generation, and increases nuclear Nrf2 accumulation, ARE-luciferase reporter activity and HO-1 expression

HO-1 is well-known for its cytoprotective effect against oxidative stress [29] and plays a critical role in the resolution of inflammation [30]. HO-1 expression is primarily regulated by Keap1-Nrf2 system which is sensitive to oxidative and/or electrophilic stresses [31]. DHA was shown to induce early-stage ROS generation in U937 cells [32] and HO-1 expression in BV-1 microglia [33]. We demonstrated the inhibition of ICAM-1 expression by HO-1 in our previous study [23]. In the present study, DHA was found to inhibit TNF-α-induced ICAM-1 expression, and the underlying mechanisms involved were investigated. As shown in Fig. 1, an increase in cellular ROS began at 5 min in cells treated with 100 μM DHA, and a peak increase was observed at 20 min as measured by the DCF probe. In addition to ROS, changes of cellular GSH and GSSG level were determined as indicative of oxidative stress. Cellular GSH/GSSG molar ratio was decreased significantly by 100 μM DHA treatment for 15, 20 and 30 min (data not shown). Next, we attempted to verify whether DHA could activate Nrf2 in association with HO-1 up-regulation. DHA did increase protein levels of Nrf2 (Fig. 2A); however, Nrf2 mRNA expression was not affected by DHA (Fig. 2B). In parallel, DHA treatment increased Nrf2 accumulation in the nucleus as early as 0.5 h, and this accumulation was sustained until 6 h (Fig. 2C). This indicated that the increased Nrf2 nuclear accumulation by DHA was possibly due to decreased Nrf2 degradation instead of *de novo* synthesis of Nrf2. To clarify this, we assayed the effect of DHA on Nrf2 ubiquitination and proteasome activity. Both Nrf2 ubiquitination and proteasome activity were decreased with 100 μM DHA treatment (Fig. 2D, E). Afterward, we used cells transfected with luciferase reporter vectors carrying the ARE sequence of HO-1 to ascertain the specificity of DHA for this activation. DHA induced the ARE-luciferase activity in a dose-dependent manner (Fig. 2F).

As shown in Fig. 3A and B, TNF-α did not affect the protein or mRNA expression of HO-1. However, DHA pretreatment significantly enhanced both protein and mRNA levels of HO-1 in a concentration-dependent manner. Actinomycin D (Act D) pretreatment blocked both DHA-induced HO-1 mRNA and protein synthesis, whereas cycloheximide (CHX) suppressed DHA-induced HO-1 protein expression (Fig. 3C). This suggests that DHA induction of HO-1 is acting on the transcription stage.

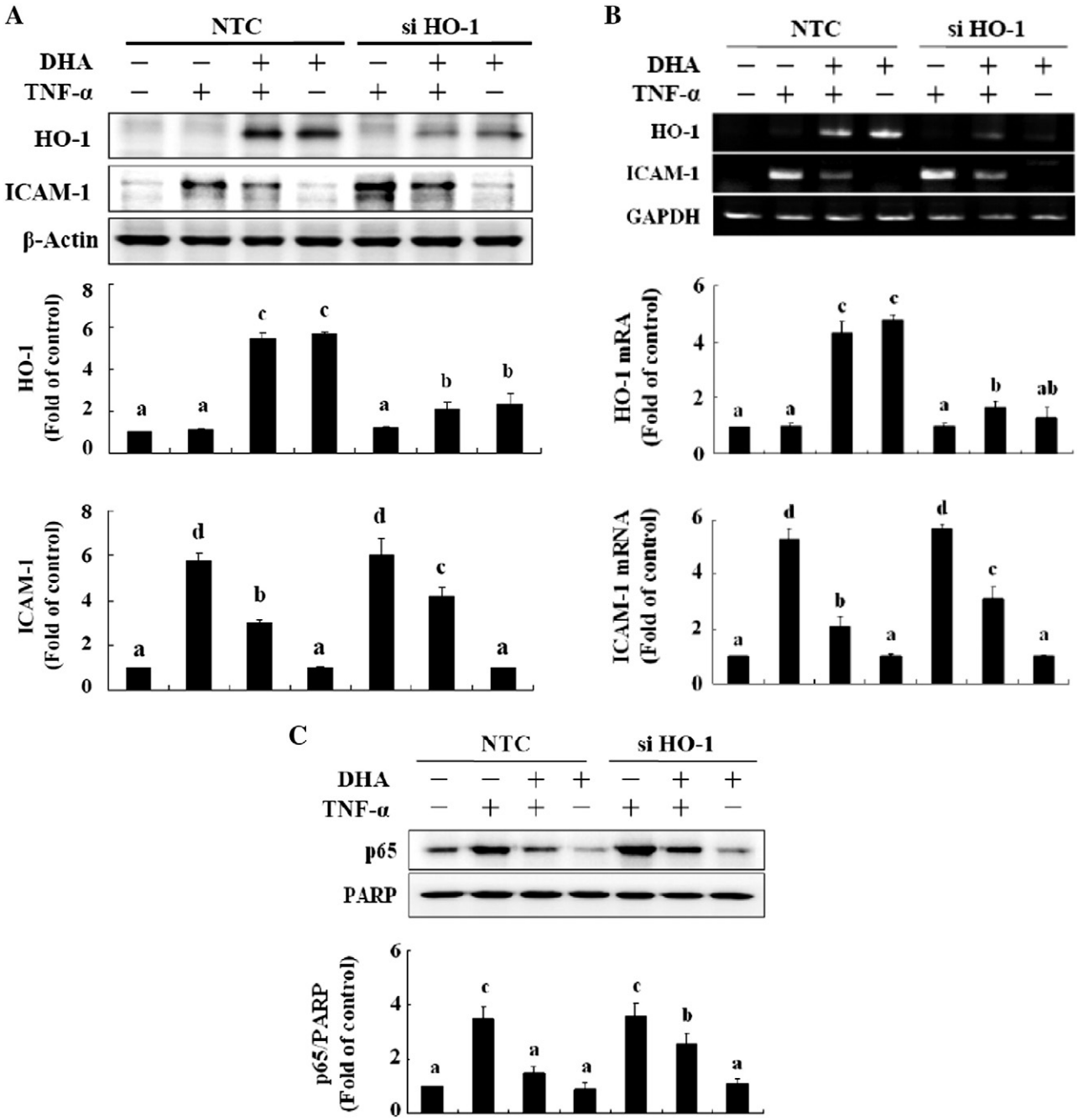

Fig. 4. Effect of HO-1 siRNA on the DHA-mediated inhibition of ICAM-1 expression and p65 translocation. An HO-1 siRNA system was used to silence HO-1 mRNA and to create a siRNA knockdown model in EA.hy926 cells. Cells were transfected with HO-1 siRNA for 8 h and then treated with 100 μM DHA for 16 h before being challenged with 1 ng/ml TNF-α for an additional 6 h. (A) Aliquots of total protein (20 μg) were used for Western blot analysis. (B) Total RNA was subjected to RT-PCR. (C) Aliquots of nuclear extract (10 μg) were used for Western blot analysis. Values are means±S.D. of three independent experiments. Values not sharing the same letter are significantly different (P <.05). NTC, nontargeting siRNA construct.

3.5. HO-1 siRNA alleviates the inhibition of TNF-α-induced ICAM-1 expression and p65 translocation by DHA

Up-regulation of HO-1 by DHA raises a possibility that DHA inhibition of TNF-α-induced ICAM-1 expression is likely associated with the induction of HO-1 expression. The role of HO-1 in the inhibition of TNF-α-induced ICAM-1 expression by DHA was confirmed by using the siRNA system. Cells were transfected with HO-1 siRNA for 8 h, followed by treatment with 100 μM DHA for 16 h and TNF-α for an additional 6 h. The efficiency of the siRNA system to silence HO-1 was ascertained by Western blot and RT-PCR assay. As shown, HO-1 siRNA partially abolished the inhibition of TNF-α-induced ICAM-1 protein (Fig. 4A, lane 3 vs. 6) and mRNA (Fig. 4B, lane 3 vs. 6) expression by DHA. Moreover, HO-1 siRNA partially abolished DHA-mediated suppression of p65 nuclear translocation (Fig. 4C, lane 3 vs. 6).

3.6. PI3K/Akt, p38 and ERK1/2 pathways are involved in DHA-induced HO-1 expression

To further elucidate the upstream signaling pathways involved in the DHA-mediated induction of HO-1, we examined the activation of PI3K/Akt and MAPKs in EA.hy926 cells. Cells were incubated with 100

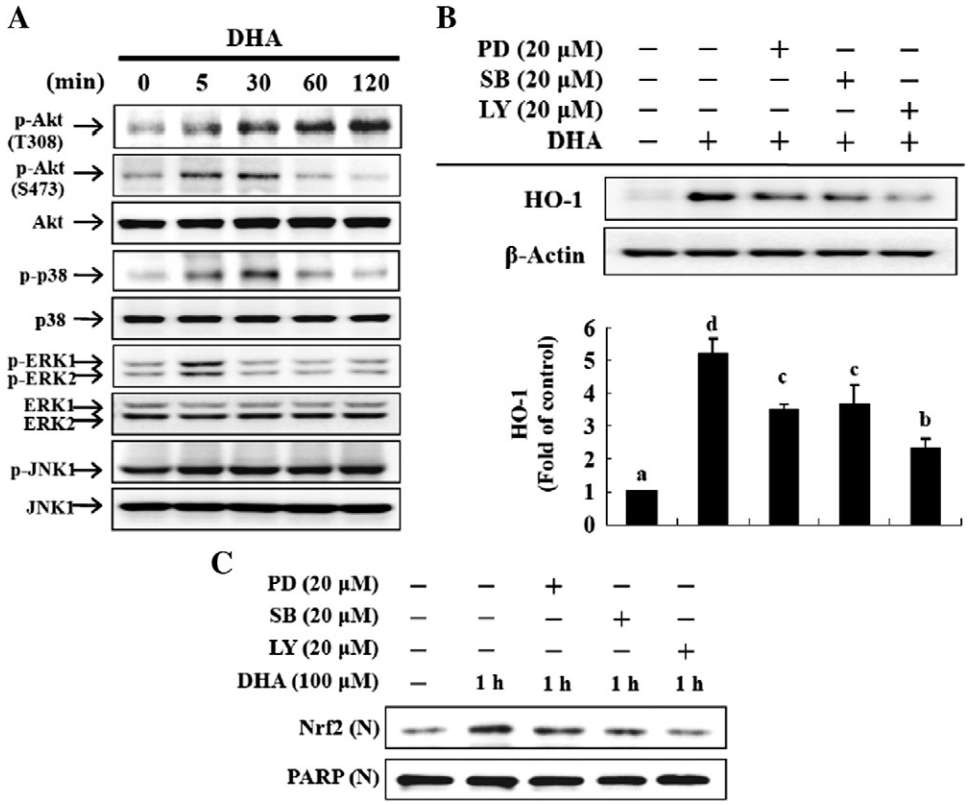

Fig. 5. PI3K/Akt, p38 and ERK1/2 pathways are involved in DHA-induced Nrf2 nuclear translocation and HO-1 expression. (A) Cells were incubated with 100 μM DHA for the indicated time periods. (B) Cells were pretreated with 20 μM PD (PD98059), SB (SB203580) and LY (LY294002) for 1 h before incubation with 100 μM DHA for another 16 h. (C) Cells were pretreated with 20 μM PD (PD98059), SB (SB203580) and LY (LY294002) for 1 h before incubation with 100 μM DHA for another 1 h. Nuclear extract [N] from cells was prepared. Values are means±S.D. of three independent experiments. Values not sharing the same letter are significantly different ($P<.05$).

μM DHA for the indicated time periods. As shown in Fig. 5A, phosphorylation of Akt, p38 and ERK1/2 was enhanced in DHA-treated cells.

To corroborate the role of individual Akt and MAPKs pathways, we examined the effects of LY294002, PD98059 and SB203580, specific inhibitors of the PI3K/Akt, ERK and p38 pathways [34,35], respectively, on DHA-induced HO-1 expression and Nrf2 nuclear translocation. As shown, inhibitors of the ERK, p38 and PI3K/Akt pathways significantly reduced DHA-induced HO-1 expression (Fig. 5B) and Nrf2 nuclear translocation (Fig 5C). No cytotoxicity determined by MTT assay was observed in cells co-treated with 100 μM DHA and each of kinase inhibitors (data not shown).

4. Discussion

Accumulating evidence indicates that dietary n-3 PUFAs may help to reduce atherogenesis [16]; however, the molecular mechanisms underlying the antiatherosclerotic effect of n-3 PUFAs have not been fully clarified. The antiatherogenic properties of fish oil were thought to be due to its capacity to inhibit the adhesion molecule expression and cytokine production induced by inflammatory stimuli [36]. Furthermore, Casós et al. reported that in apoE$^{-/-}$ mice fed a diet rich in fish oil, the reduction of atherosclerotic lesions was associated with decreased expression of endothelial adhesion molecules and NF-κB activation [37]. Some clinical studies have shown that intake of fish oil decreases plasma levels of soluble ICAM-1 and P-selectin in patients with atherosclerosis [16]. In the present study, we used DHA to represent the fish oil source and explored the mechanism(s) involved in the inhibition of TNF-α-induced ICAM-1 expression in EA.hy926 cells. Our results showed that DHA effectively inhibited TNF-α-induced inflammatory responses in EA.hy926 cells and that this suppression was likely associated with an up-regulation of Nrf2-dependent HO-1 and a down-regulation of the IKK/NF-κB signaling pathway.

Vascular inflammation plays a key role in the pathogenesis of atherosclerosis, and the importance of adhesion molecules in atherosclerosis has been examined in many studies. For example, knockout of ICAM-1 results in attenuation of atherosclerotic plaques in apoE knockout mice [38], and levels of soluble adhesion molecules have been implicated as risk predictors for cardiovascular events [16]. These observations collectively indicate that regulation of adhesion molecule expression (e.g., ICAM-1) is a potential target for the development of new therapeutics in the prevention and treatment of atherosclerosis. We showed here that TNF-α-induced ICAM-1 expression and ICAM-1 luciferase reporter activity were inhibited by DHA pretreatment (Supplemental Fig. 1A, B, C). Moreover, DHA pretreatment inhibited the adhesion of HL-60 cells to activated EA.hy926 cells (Supplemental Fig. 1D). Thus, DHA has the potential to prevent vascular inflammation [13,14].

The essential role of the NF-κB pathway in the TNF-α-induced expression of adhesion molecules has been convincingly demonstrated by a previous study [3], and disruption of NF-κB activation has been shown to delay or prevent atherogenesis [39]. As described earlier, the dissociation of NF-κB from IκB requires phosphorylation of IκB, which results in rapid and ubiquitous degradation of IκB. A study by Denk et al. [40] showed that overexpressing dominant negative IκB, which is resistant to proteolysis, completely blocked the TNF-α-induced expression of ICAM-1, VCAM-1 and E-selectin. Kaileh et al. [41] recently reported that withaferin A inhibited TNF-α-stimulated NF-κB activation by blocking the activity of IKKβ kinase and IκB degradation. Our data indicated that DHA inhibited not only the

upstream IKK and IκBα phosphorylation but also IκBα degradation (Supplemental Fig. 2A, B). In addition, we found that TNF-α-induced NF-κB and DNA binding activity and nuclear translocation of p65 were attenuated in response to DHA pretreatment (Supplemental Fig. 2C, D). This provides evidence that DHA antagonizes adhesion molecule expression in TNF-α-activated EA.hy926 cells by attenuating the NF-κB signaling pathway.

Induction of antioxidant enzymes is associated with health benefit. Among the antioxidant enzymes, HO-1 expression is considered to be an adaptive and beneficial response to oxidative stress in various cells [11,42]. HO-1 expression is primarily regulated by Keap1-Nrf2 system which is oxidative stress sensitive [31]. In this study, DHA triggered early-stage and transient ROS generation (Fig. 1). A similar result was found in a previous study [32]. Aires et al. [32] found that a significant increase in ROS was observed after 1 min of treatment with DHA in U937 cells and that the level of ROS was decreased as a function of time thereafter, up to 1 h of incubation.

Recent evidence suggests that Nrf2 is required for the activation of the HO-1 gene [11]. In this study, DHA enhanced Nrf2 nuclear translocation and ARE-luciferase activity (Fig. 2C,F). Moreover, DHA increased total cellular Nrf2 level, but no effect on Nrf2 mRNA was noted (Fig. 2B). We determined the effect of DHA on the chymotrypsin-like proteasome activity and found that DHA decreased ubiquitinated Nrf2 (Fig. 2D) and inhibited the chymotrypsin-like proteasome activity (Fig. 2E). Thus, our results suggest that the increased Nrf2 activation by DHA was likely associated with increased Nrf2 nuclear translocation by decreasing Nrf2 ubiquitination and proteasome degradation. To our knowledge, this is the first study that demonstrates that DHA may regulate the ubiquitination and proteasomal degradation of Nrf2 and alter its cellular localization.

In this study, DHA dose-dependently up-regulated HO-1 mRNA and protein expression in EA.hy926 cells (Figs. 3A,B), and the induction of the HO-1 gene by DHA was primarily regulated at the transcriptional level (Fig. 3C). HO-1 has anti-inflammatory endothelial protective action via reduction of TNF-α-induced expression of various adhesion molecules [43]. Several anti-inflammatory or anticarcinogenic phytochemicals, e.g., phenethyl isothiocyanate, sulforaphane and curcumin, suppress NF-κB signaling and activate the Nrf2-ARE pathway [44], which suggests that NF-κB and Nrf2 may cross-talk with each other. These findings suggest the possibility that DHA inhibits TNF-α-induced ICAM-1 expression via suppression of NF-κB activation, which is regulated by HO-1 induction. We confirmed the importance of HO-1 in the inhibition of ICAM-1 expression by using HO-1 siRNA. In cells transfected with siHO-1, the inhibition of TNF-α-induced ICAM-1 protein and mRNA expression by DHA was partially abolished (Fig. 4A, B). We also ascertained by transfection with HO-1 siRNA that HO-1 inhibited ICAM-1 expression through attenuation of NF-κB activation (Fig. 4C). These findings suggest that the cytoprotective actions of HO-1 were at least partially attributed to the attenuation of ICAM-1 expression, and NF-κB appeared to be the target. Taken together, DHA inhibited TNF-α-induced ICAM-1 expression likely via two distinct mechanisms: inhibition of the IKK/NF-κB signaling pathway and activation of the Nrf2/HO-1 pathway. There is cross-talk between these two mechanisms. Li et al. [44] reported that activation of Nrf2 antioxidant signaling occurs concomitantly with attenuation of NF-κB inflammatory response in the face of sulforaphane, phenethyl isothiocyanate and curcumin treatments.

Previous studies have suggested that several signaling pathways, including MAPKs [45], PKC [46] and PI3K pathways [47], are involved in Nrf2 activation and subsequent HO-1 induction. Among these, the contribution of PI3K/Akt and MAPKs in the regulation of HO-1 in various cell types is well appreciated [45,47]. Our results supported the role of PI3K/Akt, ERK1/2 and p38 in DHA-induced HO-1 expression (Fig. 5A, B), and their action was via changes in DHA-induced Nrf2 nuclear translocation (Fig. 5C). The Keap1–Nrf2 complex is the closest ROS receptor in mammals, and modification

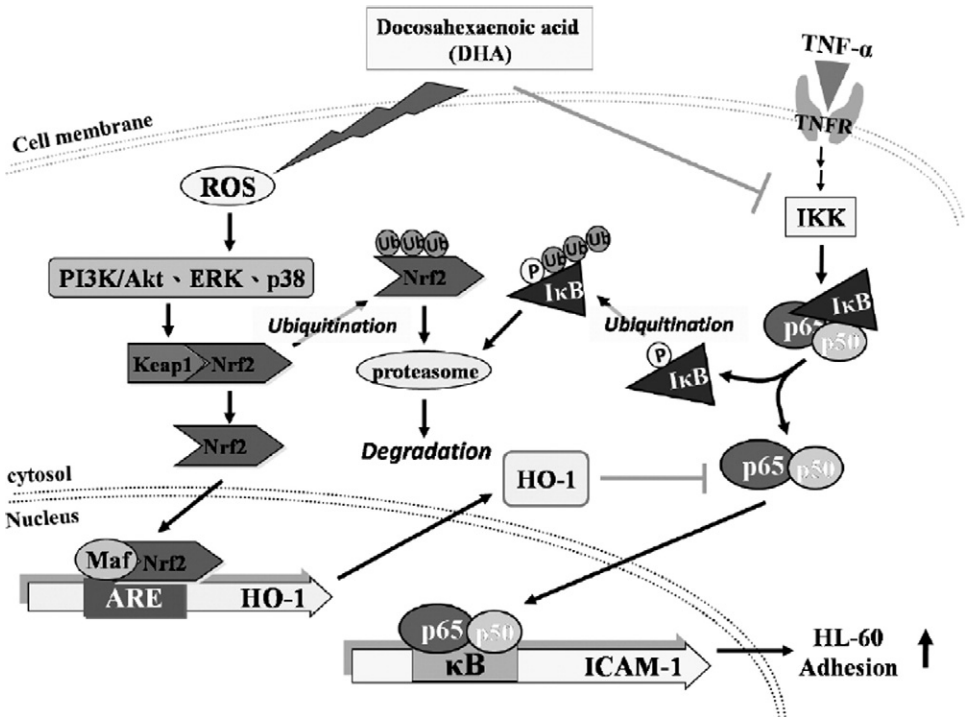

Fig. 6. Scheme summarizing the pathways that mediate the inhibition of expression of ICAM-1 and adhesion of HL-60 cells to EA.hy926 cells by DHA under inflammatory conditions. DHA causes early-stage and transient generation of ROS and activates PI3K/Akt, ERK1/2 and p38 signaling pathways, the dissociation of Nrf2 from Keap1 and its nuclear translocation. Nrf2 then binds to the ARE, which leads to an induction of HO-1 expression and inhibits ICAM-1 expression and subsequent HL-60 cell adhesion. DHA also inhibits TNF-α-induced IKK/NF-κB activation, ICAM-1 expression and eventual HL-60 cell adhesion. There is cross-talk between HO-1 and NF-κB.

of Keap1 is proposed to affect Nrf2 stability and nuclear accumulation [48]. ROS cause Keap1 modification through different pathways including cysteine oxidation; Zn release; and phosphorylation by PKC, PI3K and ERK [48]. In this study, 100 μM DHA increased cellular ROS early at 5 min, and the peak effect was observed at 20 min after DHA treatment (Fig. 1). Thus, the early-stage and transient generation of ROS by DHA might activate the signaling pathways that lead to Nrf2 nuclear translocation and eventual HO-1 induction.

The findings of this study are schematically presented in Fig. 6. In summary, DHA significantly inhibits TNF-α-induced ICAM-1 mRNA and protein expression and the subsequent adhesion of HL-60 cells to activated EA.hy926 cells. Two working mechanisms by which DHA inhibits ICAM-1 expression are identified: one is inhibition of the IKK/NF-κB pathway, and the other is activation of the Nrf2/HO-1 pathway. There is cross-talk between these two mechanisms. Our results support a beneficial role of DHA in anti-inflammation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2012.05.003.

References

[1] Blake GJ, Ridker PM. Novel clinical markers of vascular wall inflammation. Circ Res 2001;89:763–71.
[2] Lusis AJ. Atherosclerosis. Nature 2000;407:233–41.
[3] Oh JH, Park EJ, Park JW, Lee J, Lee SH, Kwon TK. 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 2010;10:572–9.
[4] Karin M, Delhase M. The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin Immunol 2000;12:85–98.
[5] Farombi EO, Surh YJ. Heme oxygenase-1 as a potential therapeutic target for hepatoprotection. J Biochem Mol Biol 2006;39:479–91.
[6] Idriss NK, Blann AD, Lip GY. Hemoxygenase-1 in cardiovascular disease. J Am Coll Cardiol 2008;52:971–8.
[7] Paine A, Eiz-Vesper B, Blasczyk R, Immenschuh S. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. Biochem Pharmacol 2010;80:1895–903.
[8] Ishikawa K, Sugawara D, Goto J, Watanabe Y, Kawamura K, Shiomi M, et al. Heme oxygenase-1 inhibits atherogenesis in Watanabe heritable hyperlipidemic rabbits. Circulation 2001;104:1831–6.
[9] Ishikawa K, Sugawara D, Wang X, Suzuki K, Itabe H, Maruyama Y, et al. Heme oxygenase-1 inhibits atherosclerotic lesion formation in LDL-receptor knockout mice. Circ Res 2001;88:506–12.
[10] Yet SF, Layne MD, Liu X, Chen YH, Ith B, Sibinga NE, et al. Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. FASEB J 2003;17:1759–61.
[11] Alam J, Cook JL. Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des 2003;9:2499–511.
[12] Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med 2009;47:1304–9.
[13] Mullen A, Loscher CE, Roche HM. Anti-inflammatory effects of EPA and DHA are dependent upon time and dose–response elements associated with LPS stimulation in THP-1-derived macrophages. J Nutr Biochem 2010;21:444–50.
[14] Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr 2002;21:495–505.
[15] Ghosh-Choudhury T, Mandal CC, Woodruff K, St Clair P, Fernandes G, Choudhury GG, et al. Fish oil targets PTEN to regulate NFkappaB for downregulation of anti-apoptotic genes in breast tumor growth. Breast Cancer Res Treat 2009;118:213–28.
[16] Paulo MC, Andrade AM, Andrade ML, Morais MG, Kiely M, Parra D, et al. Influence of n-3 polyunsaturated fatty acids on soluble cellular adhesion molecules as biomarkers of cardiovascular risk in young healthy subjects. Nutr Metab Cardiovasc Dis 2008;18:664–70.
[17] Brown AA, Hu FB. Dietary modulation of endothelial function: implications for cardiovascular disease. Am J Clin Nutr 2001;73:673–86.
[18] Chen H, Li D, Chen J, Roberts GJ, Saldeen T, Mehta JL. EPA and DHA attenuate ox-LDL-induced expression of adhesion molecules in human coronary artery endothelial cells via protein kinase B pathway. J Mol Cell Cardiol 2003;35:769–75.
[19] Mayer K, Merfels M, Muhly-Reinholz M, Gokorsch S, Rosseau S, Lohmeyer J, et al. Omega-3 fatty acids suppress monocyte adhesion to human endothelial cells: role of endothelial PAF generation. Am J Physiol Heart Circ Physiol 2002;283:H811–8.
[20] von Schacky C. n-3 PUFA in CVD: influence of cytokine polymorphism. Proc Nutr Soc 2007;66:166–70.
[21] Spector AA. Structure and lipid binding properties of serum albumin. Methods Enzymol 1986;128:320–9.
[22] Liao BC, Hsieh CW, Liu YC, Tzeng TT, Sun YW, Wung BS. Cinnamaldehyde inhibits the tumor necrosis factor-alpha-induced expression of cell adhesion molecules in endothelial cells by suppressing NF-kappaB activation: effects upon IkappaB and Nrf2. Toxicol Appl Pharmacol 2008;229:161–71.
[23] Yu AL, Lu CY, Wang TS, Tsai CW, Liu KL, Cheng YP, et al. Induction of heme oxygenase 1 and inhibition of tumor necrosis factor alpha-induced intercellular adhesion molecule expression by andrographolide in EA.hy926 cells. J Agric Food Chem 2010;58:7641–8.
[24] Cheng WL, Lii CK, Chen HW, Lin TH, Liu KL. Contribution of conjugated linoleic acid to the suppression of inflammatory responses through the regulation of the NF-kappaB pathway. J Agric Food Chem 2004;52:71–8.
[25] van de Stolpe A, Caldenhoven E, Stade BG, Koenderman L, Raaijmakers JA, Johnson JP, et al. 12-O-tetradecanoylphorbol-13-acetate- and tumor necrosis factor alpha-mediated induction of intercellular adhesion molecule-1 is inhibited by dexamethasone. Functional analysis of the human intercellular adhesion molecular-1 promoter. J Biol Chem 1994;269:6185–92.
[26] Stangl V, Lorenz M, Meiners S, Ludwig A, Bartsch C, Moobed M, et al. Long-term up-regulation of eNOS and improvement of endothelial function by inhibition of the ubiquitin-proteasome pathway. FASEB J 2004;18:272–9.
[27] Woo CH, Eom YW, Yoo MH, You HJ, Han HJ, Song WK, et al. Tumor necrosis factor-alpha generates reactive oxygen species via a cytosolic phospholipase A2-linked cascade. J Biol Chem 2000;275:32357–62.
[28] Kisselev AF, Goldberg AL. Proteasome inhibitors: from research tools to drug candidates. Chem Biol 2001;8:739–58.
[29] Abraham NG, Kappas A. Heme oxygenase and the cardiovascular–renal system. Free Radic Biol Med 2005;39:1–25.
[30] Pae HO, Lee YC, Chung HT. Heme oxygenase-1 and carbon monoxide: emerging therapeutic targets in inflammation and allergy. Recent Pat Inflamm Allergy Drug Discov 2008;2:159–65.
[31] Kobayashi A, Kang MI, Watai Y, Tong KI, Shibata T, Uchida K, et al. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mol Cell Biol 2006;26:221–9.
[32] Aires V, Hichami A, Filomenko R, Plé A, Rébé C, Bettaieb A, et al. Docosahexaenoic acid induces increases in [Ca2+]i via inositol 1,4,5-triphosphate production and activates protein kinase C gamma and -delta via phosphatidylserine binding site: implication in apoptosis in U937 cells. Mol Pharmacol 2007;72:1545–56.
[33] Lu DY, Tsao YY, Leung YM, Su KP. Docosahexaenoic acid suppresses neuroinflammatory responses and induces heme oxygenase-1 expression in BV-2 microglia: implications of antidepressant effects for ω-3 fatty acids. Neuropsychopharmacology 2010;35:2238–48.
[34] Lu CY, Li CC, Lii CK, Yao HT, Liu KL, Tsai CW, et al. Andrographolide-induced pi class of glutathione S-transferase gene expression via PI3K/Akt pathway in rat primary hepatocytes. Food Chem Toxicol 2011;49:281–9.
[35] Chang HC, Chen HW, Tung HS, Liu KL, Tsai CW, Lii CK. Butein up-regulates the expression of the pi class of glutathione *S*-transferase in rat primary hepatocytes through the ERK/AP-1 pathway. J Agric Food Chem 2010;58:8994–9000.
[36] Chen W, Esselman WJ, Jump DB, Busik JV. Anti-inflammatory effect of docosahexaenoic acid on cytokine-induced adhesion molecule expression in human retinal vascular endothelial cells. Invest Ophthalmol Vis Sci 2005;46:4342–7.
[37] Casos K, Saiz MP, Ruiz-Sanz JI, Mitjavila MT. Atherosclerosis prevention by a fish oil-rich diet in apoE(−/−) mice is associated with a reduction of endothelial adhesion molecules. Atherosclerosis 2008;201:306–17.
[38] Bourdillon MC, Poston RN, Covacho C, Chignier E, Bricca G, McGregor JL. ICAM-1 deficiency reduces atherosclerotic lesions in double-knockout mice (ApoE(−/−)/ICAM-1(−/−)) fed a fat or a chow diet. Arterioscler Thromb Vasc Biol 2000;20:2630–5.
[39] Lopez-Franco O, Hernandez-Vargas P, Ortiz-Munoz G, Sanjuan G, Suzuki Y, Ortega L, et al. Parthenolide modulates the NF-kappaB-mediated inflammatory responses in experimental atherosclerosis. Arterioscler Thromb Vasc Biol 2006;26:1864–70.
[40] Denk A, Goebeler M, Schmid S, Berberich I, Ritz O, Lindemann D, et al. Activation of NF-kappa B via the Ikappa B kinase complex is both essential and sufficient for proinflammatory gene expression in primary endothelial cells. J Biol Chem 2001;276:28451–8.
[41] Kaileh M, Vanden Berghe W, Heyerick A, Horion J, Piette J, Libert C, et al. Withaferin A strongly elicits IkappaB kinase beta hyperphosphorylation concomitant with potent inhibition of its kinase activity. J Biol Chem 2007;282:4253–64.
[42] Takahashi T, Morita K, Akagi R, Sassa S. Heme oxygenase-1: a novel therapeutic target in oxidative tissue injuries. Curr Med Chem 2004;11:1545–61.
[43] Soares MP, Seldon MP, Gregoire IP, Vassilevskaia T, Berberat PO, Yu J, et al. Heme oxygenase-1 modulates the expression of adhesion molecules associated with endothelial cell activation. J Immunol 2004;172:3553–63.
[44] Li W, Khor TO, Xu C, Shen G, Jeong WS, Yu S, et al. Activation of Nrf2-antioxidant signaling attenuates NFkappaB-inflammatory response and elicits apoptosis. Biochem Pharmacol 2008;76:1485–9.
[45] Gong P, Hu B, Cederbaum AI. Diallyl sulfide induces heme oxygenase-1 through MAPK pathway. Arch Biochem Biophys 2004;432:252–60.
[46] Numazawa S, Ishikawa M, Yoshida A, Tanaka S, Yoshida T. Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress. Am J Physiol Cell Physiol 2003;285:C334–42.
[47] Hwang YP, Jeong HG. Mechanism of phytoestrogen puerarin-mediated cytoprotection following oxidative injury: estrogen receptor-dependent up-regulation of PI3K/Akt and HO-1. Toxicol Appl Pharmacol 2008;233:371–81.
[48] D'Autreaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat Rev Mol Cell Biol 2007;8:813–24.