17-Oxo-docosahexaenoic acid induces Nrf2-mediated expression of heme oxygenase-1 in mouse skin *in vivo* and in cultured murine epidermal cells

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ABSTRACT

Recently, growing attention has been given to new classes of bioactive lipid mediators derived from ω-3 polyunsaturated fatty acids, such as docosahexaenoic acid (DHA), especially in the context of their role as endogenous signal modulators. One such molecule is 17-oxo-DHA, generated from DHA by the action of COX2 and a dehydrogenase. The redox-sensitive transcription factor, Nrf2 plays a key role in cellular stress responses. In the present study, the effects of 17-oxo-DHA on Nrf2-mediated expression of cytoprotective enzymes were examined in mouse skin *in vivo* and cultured murine epidermal JB6 cells. Topical application of 17-oxo-DHA markedly elevated the nuclear localization of Nrf2 and expression of heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase-1 in hairless mouse skin. In contrast to 17-oxo-DHA, the non-electrophilic metabolic precursor 17-hydroxy-DHA was a much weaker inducer of Nrf2 activation and its target protein expression. Likewise, 17-oxo-DHA significantly enhanced nuclear translocation and transcriptional activity of Nrf2 with concomitant upregulation of HO-1 expression in cultured JB6 cells. 17-Oxo-DHA was a much stronger inducer of Nrf2-mediated antioxidant response than its parent molecule, DHA. HO-1 expression was abolished in Nrf2 knockdown JB6 cells or embryo fibroblasts from Nrf2 knock out mice. 17-Oxo-DHA also markedly reduced the level of Keap1 protein by inducing ubiquitination. Mutation of Cys151 and Cys273 in Keap1 abrogated 17-oxo-DHA-induced ubiquitination and proteasome-mediated degradation of Keap1 as well as HO-1 expression, suggesting that these cysteine residues are putative sites for 17-oxo-DHA binding. Further, Keap1 degradation stimulated by 17-oxo-DHA coincided with accumulation of the autophagy substrate, p62/SQSTM1.

1. Introduction

Skin is constantly exposed to solar ultraviolet (UV) radiation and other external oxidative stress stimuli, which generate reactive oxygen species (ROS) [1]. The loss of redox homeostasis as a consequence of excessive intracellular accumulation of ROS and/or reduced capacity of antioxidant defence contributes to pathogenesis of skin carcinogenesis and dermatitis as well as skin aging [2–4]. When cellular anti-oxidant defense capacity is overwhelmed by ROS, the self-correcting adaptive physiological response reestablishes homeostasis by inducing a cytoprotective program [5]. A significant attention has been given to the

wide array of natural or synthetic agents that can prevent the harmful effects of ROS by restoring normal redox homeostasis.

Cellular redox homeostasis is maintained by the constitutive induction of genes that encode a distinct set of antioxidant and other cytoprotective proteins [6]. These include heme oxygenase-1 (HO-1) and NAD(P)H: quinoneoxidoreductase-1 (NQO1) that are involved in cellular defense against oxidative stress [7]. Thus, HO-1 and NQO-1 knockout mice were more prone to skin tumor development [8,9]. The robust induction of aforementioned antioxidant proteins is known to be mediated by a principle redox regulator, nuclear factor erythroid 2-related factor 2 (Nrf2) [10,11].

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Under unstressed conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex with Kelch-like ECH-associated protein 1 (Keap1) [12,13]. By interacting with Nrf2, Keap1 enhances degradation of Nrf2 with the help of Cul3 ubiquitin ligase complex [14–16]. Cul3-mediated ubiquitination is switched from Nrf2 to Keap1 in response to an oxidative or electrophilic stimulus, which facilitates the subsequent degradation of Keap1 [17,18]. As a result, a Keap1-Nrf2 complex is disrupted, and the Keap1-directed Nrf2 degradation is impeded. Once dissociated from Keap1, Nrf2 translocates into the nucleus where it binds to antioxidant response element (ARE) or electrophile response element (EpRE) to drive the transcription of many antioxidant and related stress-responsive genes [10,19].

Health beneficial effects of long chain omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been corroborated by results from various laboratory, population-based and clinical studies [20–22]. n-3 PUFAs possess anti-oxidative and anti-inflammatory properties [23–25]. A representative n-3 PUFA abundant in the fish oil is docosahexaenoic acid (DHA). Results from previous studies clearly demonstrate that dietary supplementation of DHA alleviates symptoms of diverse pathological conditions linked to oxidative stress and inflammation [26,27].

Groeger et al. have identified a new class of n-3 electrophilic fatty acid oxo-derivatives (EFOXs) that are generated in activated macrophages, neutrophils and some animal tissues, underscoring their potential as bioactive lipid mediators capable of modulating several cellular signaling pathways including Nrf2-dependent anti-oxidant response [28]. The formation of these reactive species was dependent on cyclooxygenase-2 (COX-2), especially in the presence of aspirin. In this study, we have focused on 17-oxo-DHA, a mono-oxygenated electrophilic metabolite of DHA. Owing to endogenous nature together with its potential to simultaneously stimulate multiple cytoprotective pathways [29–31], 17-oxo-DHA may contribute, at least in part, to the protective effects of DHA against oxidative stress as well as inflammation-associated disorders. In this study, we examined whether 17-oxo-DHA could exert anti-oxidative effects by activating the Nrf2 and subsequently upregulating expression of its target proteins in mouse skin *in vivo* and cultured mouse skin epidermal cells.

2. Methods

2.1. Chemicals and reagents

DHA (purity > 98%), 17-oxo-DHA (17-keto-4(Z),7(Z),10(Z),13(Z),15(E),19(Z)-docosahexaenoic acid), and 17-hydroxy-DHA (17-hydroxy-4Z,7Z,10Z,13Z,13Z,15E,19Z-docosahexaenoic acid) were supplied by Cayman Chemical Co. (Ann Arbor, MI, USA). Primary antibodies for Nrf2 and Keap1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal HO-1 antibody was a product of Abcam (Cambridge, UK) or Stressgen (Ann Arbor, MI, USA). Antibody for NQO-1 was procured from Abcam (Cambridge, UK) and Enzo Life Sciences (Farmingdale, NY, USA). Primary antibodies for lamin B and α-tubulin were the product of Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce Biotechnology (Rockford, IL, USA). The bicinchoninic acid (BCA) protein assay reagent was supplied by Pierce Biotechnology (Rockford, IL, USA). Antibody for β-actin was provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyvinylidene difluoride (PVDF) membrane was obtained from Gelman Laboratory (Ann Arbor, MI, USA). The enhanced chemiluminescent detection reagent was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL, USA). Eagle's Minimum Essential Media (MEM), fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). The Nrf2 siRNA (5′-UUCUGUCGCUGACUAAAGUCAAACA-3′) and Stealth™ universal RNAi negative control duplexes were purchased from Invitrogen (Carlsbad, CA, USA). The plasmid pH015-luc was kindly provided by Dr. J. Alam from Alton Ochsner Medical Foundation (New Orleans, LA, USA).

2.2. Animal treatment

Female HR-1 hairless mice (5–6 weeks of age) were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). Animals were kept in climate-controlled quarters (24 °C at 50% humidity) with a 12-h light/12-h dark cycle. DHA (10 μmol), 17-oxo-DHA (20 nmol), and 17-OH-DHA (20 nmol) were dissolved in 200 μl of acetone. These compounds were topically applied onto the dorsal skin of mice for 2.5 or 5 h. Control animals were treated with the vehicle only.

2.3. Cell culture and transient transfection

Mouse epidermal JB6 Cl 41 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in monolayers at 37 °C, 5% CO_2 by using MEM, containing 5% FBS, 1.5 mg/ml sodium bicarbonate. Cells at a confluence of 60–70% were transfected with control siRNA or Nrf2 siRNA (25 nM) with lipofectamine RNAi-MAX reagent according to the manufacturer's instructions. After 36 h transfection, cells were treated with DHA for additional 12 h, and cell lysates were prepared as described earlier.

2.4. Reverse transcrption polymerase chain reaction (RT-PCR)

Total RNA was isolated from the skin tissue or JB6 Cl 41 cells by using TRIzol® (Invitrogen; Carlsbad, CA, USA) according to the protocol supplied by the manufacturer. To generate cDNA, 1 μg of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase (Promega; Madison, WI, USA) for 50 min at 42 °C and again for 15 min at 72 °C. One μl of cDNA was amplified in sequential reactions using Maxtime PCR PreMix Kit (iNtRON Biotechnology). For *in vivo* experiment with mouse skin, the mRNA expression of *actin* (40 cycle of 94 °C for 30 s, 49 °C for 35 s, and 72 °C for 30 s), *nrf2* (40 cycles of 94 °C for 15 s, 51 °C for 15 s, and 72 °C for 30 s), and *keap1* (35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s) was checked. For cell culture studies, the mRNA expression of *nrf2* (25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), *keap1* (23 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s), *ho-1* (20 cycles of 94 °C for 30 s, 53 °C for 35 s, and 72 °C for 30 s) and *actin* (20 cycles of 94 °C for 30 s, 59 °C for 35 s, and 72 °C for 30 s) was examined.

After that, a final extension was performed for 7 min at 72 °C. The primers used for RT-PCR reactions are as follows (forward and reverse, respectively): *nrf2*, 5′-CTT TAG TCA GCG ACA GAA GGA C-3′ and 5′-AGG CAT CTT GTT TGG GAA TGT G-3′; *keap1*, 5′-GGC AGG ACC AGT TGA ACA GT-3′ and 5′-GGG TCA CCT CAC TCC AGG TA-3′; *ho-1,* 5′-TAC ACA TCC AAG CCG AGA AT-3′ and 5′-GTT CCT CTG TCA GCA TCA CC-3'; *p62*, 5'-CAG CTG TTT CGT CCG TAC CT-3' and 5'-AAA AGG CAA CCA AGT CCC CA-3'; *actin*, 5′-AGA GCA TAG CCC TCG TAG AT-3′ and 5′-CCC AGA GCA AGA GAG GTA TC-3′ (forward and reverse, respectively). Amplified products were resolved by 2% agarose gel electrophoresis, followed by staining with SYBR® green, and then photographed using fluorescence in LAS-4000.

2.5. Western blot analysis

For the preparation of the mouse epidermal protein extract, fat and dermis taken off from the harvested skin samples were kept on ice, and the fat-free epidermis was immediately placed in liquid nitrogen. The pulverized skin was homogenized on ice with a Polytron® tissue homogenizer and lysed in 1 ml ice-cold lysis buffer. Lysates were centrifuged twice at 14,000 × *g* for 30 min and 15 min. The supernatant was collected, and total protein concentration was quantified by using the BCA protein assay kit. Cell lysates (30 μg protein) were mixed with sodium dodecyl sulfate (SDS) sample loading dye and boiled for 5 min
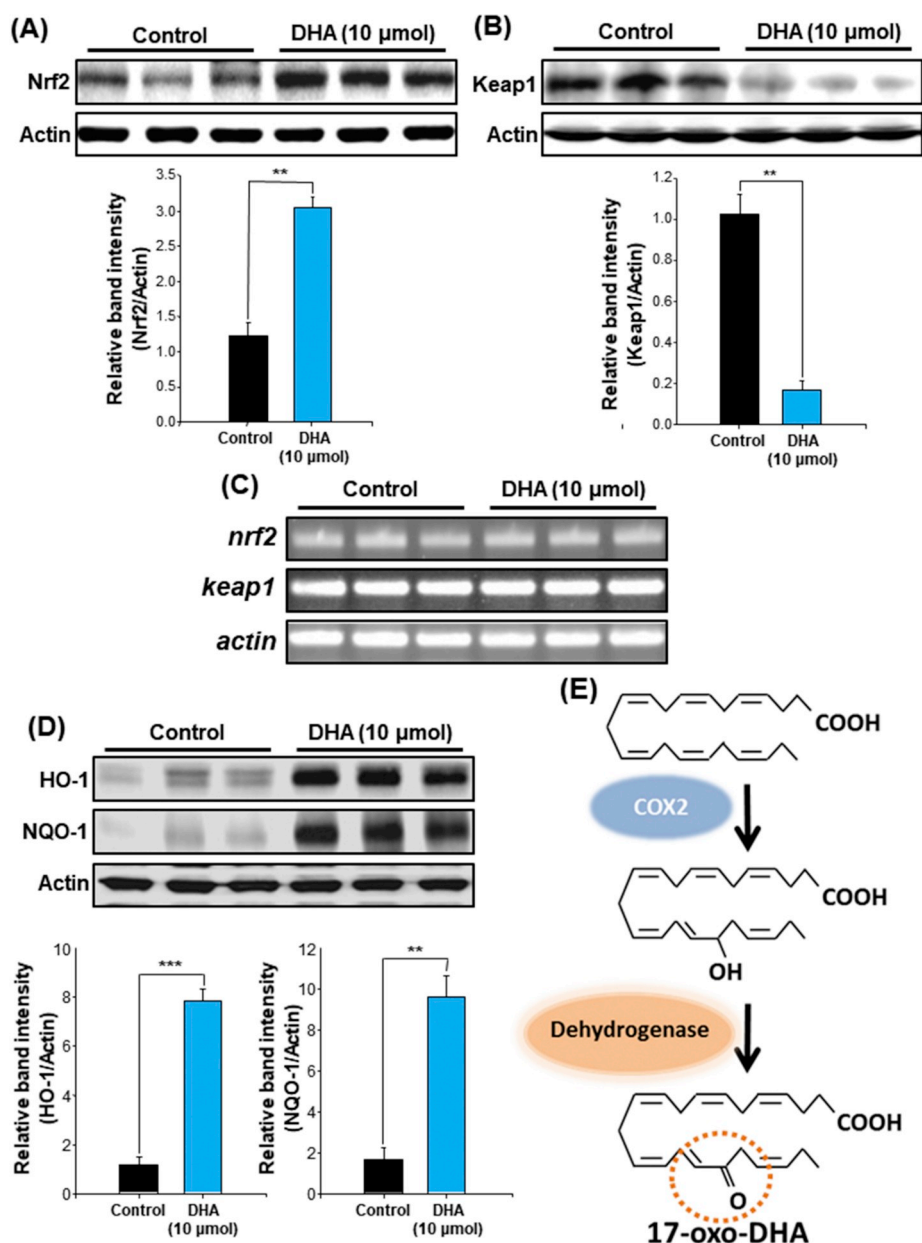
Fig. 1. Effects of DHA on the expression of Nrf2, Keap1 and its target proteins in mouse skin *in vivo*. Dorsal skin of female HR-1 hairless mice ($n = 3$ per group) was treated topically with 10 μmol of DHA. (A, B) After 2.5 h of DHA treatment, the protein levels of Nrf2 (A) and Keap1 (B) in the epidermal tissue lysates were measured by Western blot analysis as described in Methods. (C) After DHA treatment for 2.5 h, RT-PCR analysis was performed to measure mRNA expression of *nrf2, keap1*, and *actin*. (D) The expression of Nrf2 target proteins, HO-1 and NQO-1, was also measured by Western blot analysis after 4-h DHA application. Data are means ± SE. **$p < 0.01$ and ***$p < 0.001$. (E) 17-Oxo-DHA, an electrophilic derivative of DHA, is generated by COX-2 catalyzed mechanism in activated macrophages. Modulation of COX-2 activity by aspirin increases the rate of electrophilic metabolite production.

before electrophoresis on 8–15% SDS-polyacrylamide gel. After transferring to PVDF membrane, the blots were blocked with 3% fat-free dry milk-TBST (Tris-buffer saline containing 0.1% Tween 20) for 1 h at room temperature and then washed with the TBST buffer. The membranes were incubated for 4 h at room temperature with primary antibodies for HO-1 and overnight at 4 °C with primary antibodies for Nrf2, Keap1, NQO-1, actin, α-tubulin, and lamin B. Blots were washed three times with TBST at 10 min intervals followed by incubation with 1:3000 dilution of respective horseradish peroxidase conjugated secondary antibodies (rabbit, goat or mouse) for 2 h at room temperature. After blots were rinsed again three times with TBST, the immunoblots were visualized with an ECL detection kit according to the instructions provided in the protocol and visualized afterward.

2.6. Preparation of cytosolic and nuclear extracts

For homogenizing dorsal skin tissues of mice, 1 ml of buffer A [1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 2 mM KCl; 1 mM MgCl$_2$; 0.1 mM dithiothreitol (DTT); 0.1 mM EDTA; 0.1 mM phenylmethylsulfonylfluoride (PMSF)] was added. The homogenates were then centrifuged for 15 min at $18,000 \times g$; The supernatant was collected as cytosolic fraction and stored at -70 °C. The precipitated nuclei were washed once with 200 μl of buffer A plus 2 μl of 10% NP-40, centrifuged, resuspended in 200 μl of buffer C [1 mM HEPES, 5 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 100% glycerol], and subsequently centrifuged for 15 min at $21,000 \times g$. The supernatant containing nuclear extract was collected and stored at -70 °C. Finally, the concentrations of protein were checked in both cytosolic and nuclear fractions.

For the preparation of JB6 cell lysates, treated cells were gently washed with cold phosphate buffered saline (PBS), scraped and centrifuged at 4000 rpm for 5 min. Pellets were suspended in 1x cell lysis buffer enriched with protease inhibitor and incubated on ice for 1 h followed by centrifugation at $14,000 \times g$ for 15 min. Supernatant was collected as whole cell lysate. For making cytosolic and nuclear extracts, cell pellets were suspended in cold hypotonic buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.3 mM EDTA, 0.2 mM DTT, 0.1 mM PMSF], incubated for 10 min on ice and then centrifuged at $6,000 \times g$ for 5 min at 4 °C. Supernatant was collected as cytosolic extract. The residual pellets were washed with hypotonic buffer and resuspended in
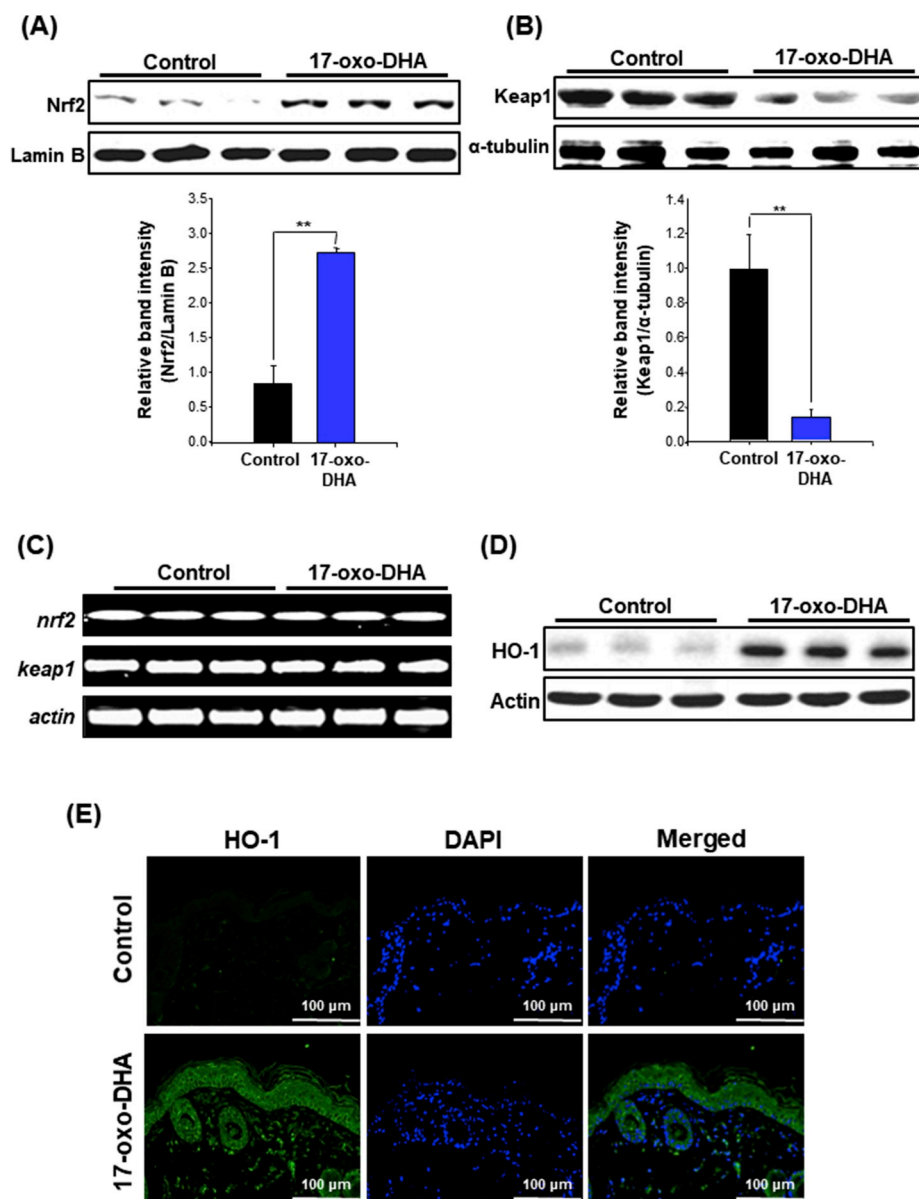
Fig. 2. Effects of 17-oxo-DHA on the Nrf2/Keap1 signaling and target protein expression in mouse skin. Dorsal skin of female HR-1 hairless mice (n = 3 per group) was treated topically with 17-oxo-DHA (20 nmol) for 2.5 h (A, B) and 5 h (C, D, E). (A, B) After 2.5 h of 17-oxo-DHA treatment, the nuclear Nrf2 (A) and cytosolic Keap1 (B) levels were assessed by Western blot analysis. (C) After 5 h of 17-oxo-DHA treatment, the mRNA expression levels of *nrf2* and *keap1* were examined by RT-PCR analysis. (D) The expression of Nrf2 and its target protein, HO-1 in the whole skin tissue lysates was measured by Western blot analysis. (E) Induction of HO-1 expression was further verified by immuno-fluorescence analysis. Data are means ± SE. **p < 0.01.

hypertonic buffer C [20 mM HEPES (pH 7.8), 25% glycerol, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF], keeping on ice for 30 min during rocking followed by centrifugation at $14,000 \times g$ for 5 min. The supernatant containing nuclear proteins was collected and stored at −70 °C. Protein concentration of whole cell lysate and cytosolic extracts were determined by using BCA protein assay kit. Protein samples from whole cell lysate, cytosolic extract or nuclear fraction were mixed with SDS sample loading dye and boiled for 5 min. Proteins were electrophoresed on SDS-polyacrylamide gel and transferred to PVDF membranes. The blots were then blocked with 5% fat-free dry milk-TBST buffer for 1 h at room temperature. The blots were incubated with primary antibodies in 3% fat-free dry milk-TBST. Following three washes with TBST, the blots were incubated with horseradish perox-idase-conjugated secondary antibodies in 3% fat-free dry milk-TBST for 1 h at room temperature. The blots were rinsed again three times with TBST, and the transferred proteins were incubated with the ECL sub-strate detection reagent for 1 min according to the manufacturer's in-structions and visualized with X-ray film in the dark room.

2.7. Immunofluorescence staining of mouse skin

Using 10% paraformaldehyde, skin tissues were fixed at room temperature. Following the permeabilization with 0.2% Triton, they were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. The tissues were then incubated with HO-1 primary antibody overnight. Alexa Fluor® conjugated secondary antibodies were used prior to mounting with 4′,6-diamidino-2-phenylindole (DAPI) for the purpose of nuclear staining.

2.8. HO-1 promoter luciferase reporter gene assay

JB6 Cl 41 cells were transfected at a confluence of 50% in 12-well plate with pHO15luc vector (0.75 μg) using Lipofectamine™ LTX for 24 h. After transfection, cells were treated with 17-oxo-DHA or DHA for additional 12 h, and cell lysate was prepared by using 1x luciferase reporter lysis buffer (Promega, Madison, WI, USA). After incubating cell lysates with a luciferase substrate, the luciferase activity was measured
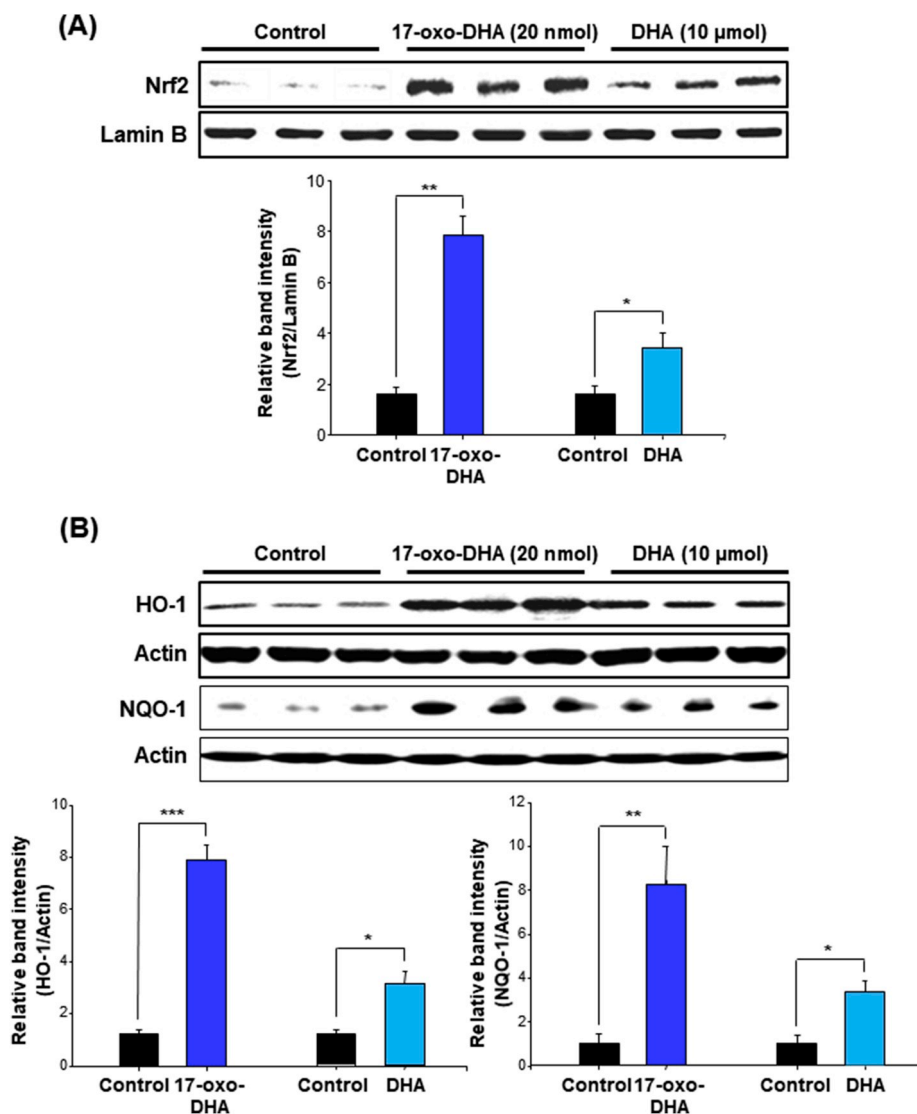
Fig. 3. Comparative effects of 17-oxo-DHA and DHA on the nuclear translocation of Nrf2 and expression of HO-1 and NQO-1 in mouse skin. Dorsal skin of female HR-1 hairless mice (*n* = 3 per group) was treated topically with 17-oxo-DHA (20 nmol) or DHA (10 μmol). (A) After 5 h of topical application, the protein levels of Nrf2 in the nuclear fraction were measured by Western blot analysis. (B) The expression of Nrf2-regulated cytoprotective proteins was determined in whole tissue lysates by Western blot analysis. Data are means ± SE. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

by the luminometer. The β-galactosidase assay was conducted according to the supplier's instructions for normalizing the luciferase activity. The relative luciferase activity was obtained by normalizing luciferase activity against β-galactosidase activity.

2.9. Immunocytochemical analysis

JB6 Cl41 cells were plated on the 8-well chamber slide and treated with 17-oxo-DHA or DHA. Cells were fixed in 95% methanol/5% distilled water for 10 min at −20 °C. After rinsing with PBST, cells were blocked for 1 h at 4 °C with fresh blocking buffer (0.1% Tween 20 in PBS, pH 7.4, containing 5% BSA) and probed with primary antibody for Nrf2 at 1:100 dilution in 5% BSA in PBST buffer overnight at 4 °C. After three washes with PBST, cells were incubated with fluorescein isothiocyanate (FITC)-goat-antimouse secondary antibody in PBST with 5% BSA for 1 h at room temperature. Cells were rinsed with PBST, and stained cells were analyzed under fluorescence microscope.

2.10. Molecular docking simulation of interaction between 17-oxo-DHA and Keap1

Covalent docking simulation was performed to predict binding pose of 17-oxo-DHA with selected reactive cysteine residues in position of mouse Keap1 protein using a CovDock module implemented in the Schrodinger program (Schrodinger, L.L.C., NY, USA). A putative mouse Keap1 structure was constructed based on human Keap1 BTB domain (PDB ID: 4CXI) and human KLKL11 BTB and BACK domains (PDB ID: 3I3N) as the BTB and IVR domains using a Prime module in the Schrodinger program. Quality control analysis by the PROCHECK program identified 92.3% residues of the model located in the most favorable regions of the Ramachandran plot.

Preparation of a protein structure was processed through "Protein Preparation Wizard" in the Schrodinger program. The minimization was terminated when the root mean square deviation of the heavy atoms in the energy minimized structure relative to the starting coordinates exceeded 0.3 Å using OPLS3e force field. The grid size for covalent docking simulation was set to 15 Å in the centroid of the

(A)

17-oxo-DHA

17-OH-DHA

(B)
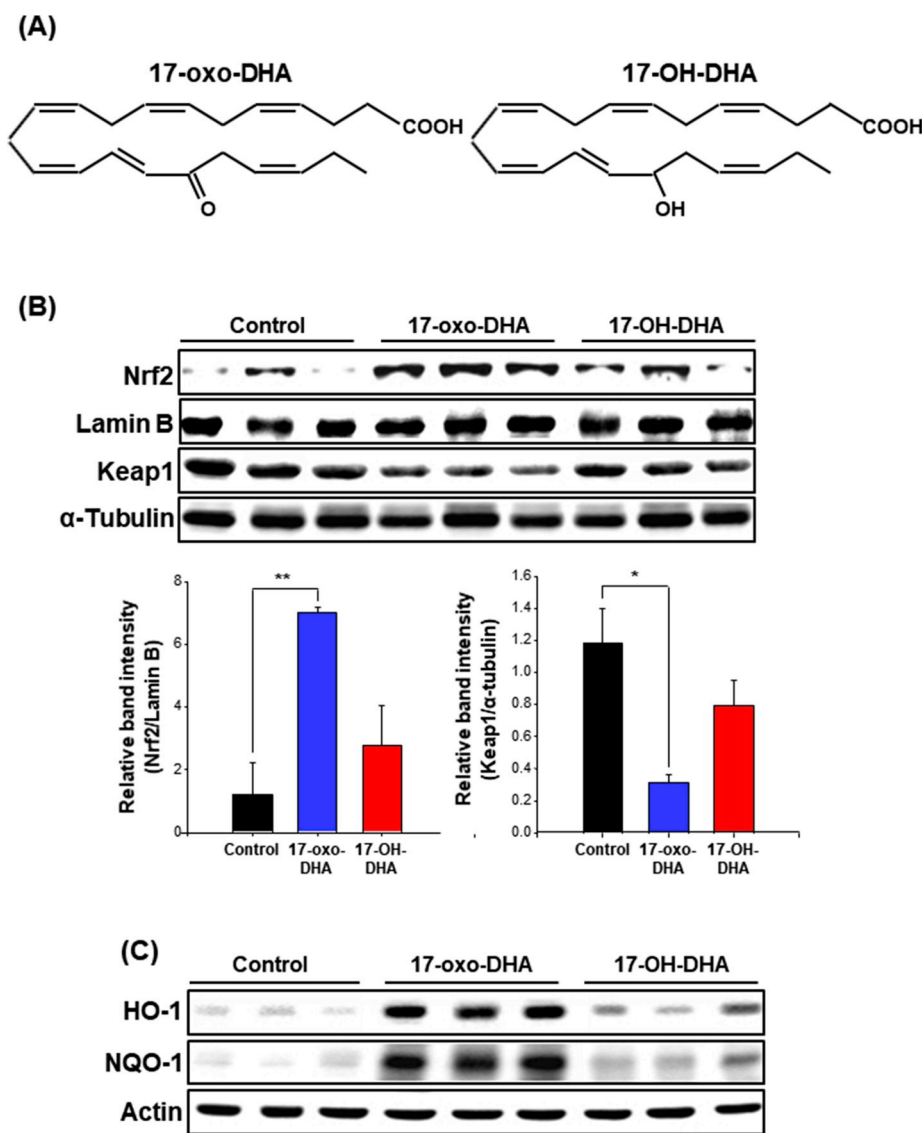
Fig. 4. The comparative effects of 17-oxo-DHA and its precursor 17-OH-DHA on Nrf2 nuclear accumulation, Keap1 degradation, and expression of HO-1 and NQO-1 in mouse skin. (A) Chemical structures of 17-oxo-DHA and its metabolic precursor 17-OH-DHA. 17-Oxo-DHA harbours an α,β-unsaturated carbonyl moiety that serves as an electrophile, whereas 17-OH-DHA is non-electrophilic. (B) Dorsal skin of female HR-1 hairless mice (*n* = 3 per group) was treated topically with 17-oxo-DHA (20 nmol) or 17-OH-DHA (20 nmol). (B) After 5 h of treatment, the protein levels of nuclear Nrf2 and cytosolic Keap1 were determined by Western blot analysis. (C) The expression of HO-1, and NQO-1 in the whole tissue lysates was also measured by Western blot analysis. Data are means ± SE. *$p < 0.05$ and **$p < 0.01$.

(C)

cysteine 151 and 273. 17-Oxo-DHA was sketched and was minimized with MMFFs force field through "LigPrep" in the Schrodinger program. Flexible ligand sampling with the SP docking mode was considered in the docking procedure. The predefined "Michael addition" reaction was used to form covalent bonds. All other parameters were set to defaults for the CovDock docking procedure.

2.11. Statistical analysis

Results were expressed as the mean ± SE or SD of at least three independent experiments. In particular, statistical significance was determined by Student's *t*-test, and the criterion for statistical significance was *p*-value < 0.05.

3. Results

3.1. DHA elevates the expression of Nrf2 and its target proteins in mouse skin in vivo

In order to determine the ability of DHA in stimulating the Nrf2-dependent antioxidant response in mouse skin, we initially examined the expression of a Nrf2 and its negative regulator, Keap1 in epidermal tissue lysates by Western blot analysis. When shaven back of female hairless C57BL/6 mice was treated topically with DHA (10 μmol) for 2.5 h, an elevated level of Nrf2 was observed compared to vehicle-treated control skin (Fig. 1A). Under the same experimental conditions, the protein expression of Keap1 was decreased (Fig. 1B). However, the mRNA expression levels of *nrf2* and *keap1* did not change in response to DHA treatment (Fig. 1C). Topical application of DHA also resulted in enhanced expression of HO-1 and NQO-1, two representative target proteins regulated by Nrf2, in mouse skin (Fig. 1D).

DHA undergoes oxidative metabolism to produce 17-oxo-DHA that has an α,β-unsaturated carbonyl moiety (Fig. 1E). Topical application of DHA onto hairless mice gave rise to the formation of 17-oxo-DHA as well as 17-OH-DHA (Seonghoon Kim and Young-Joon Surh, unpublished observation). Due to its electrophilic nature, 17-oxo-DHA is speculated to modulate intracellular redox signaling. Though this 17-oxo-DHA has been shown to activate Nrf2 in cultured macrophages and some epithelial cells [28,29], the *in vivo* effects of this EFFOX in experimental animals have not been reported yet. This prompted us to assess its capability to activate Nrf2-mediated antioxidant response in mouse skin *in vivo*.
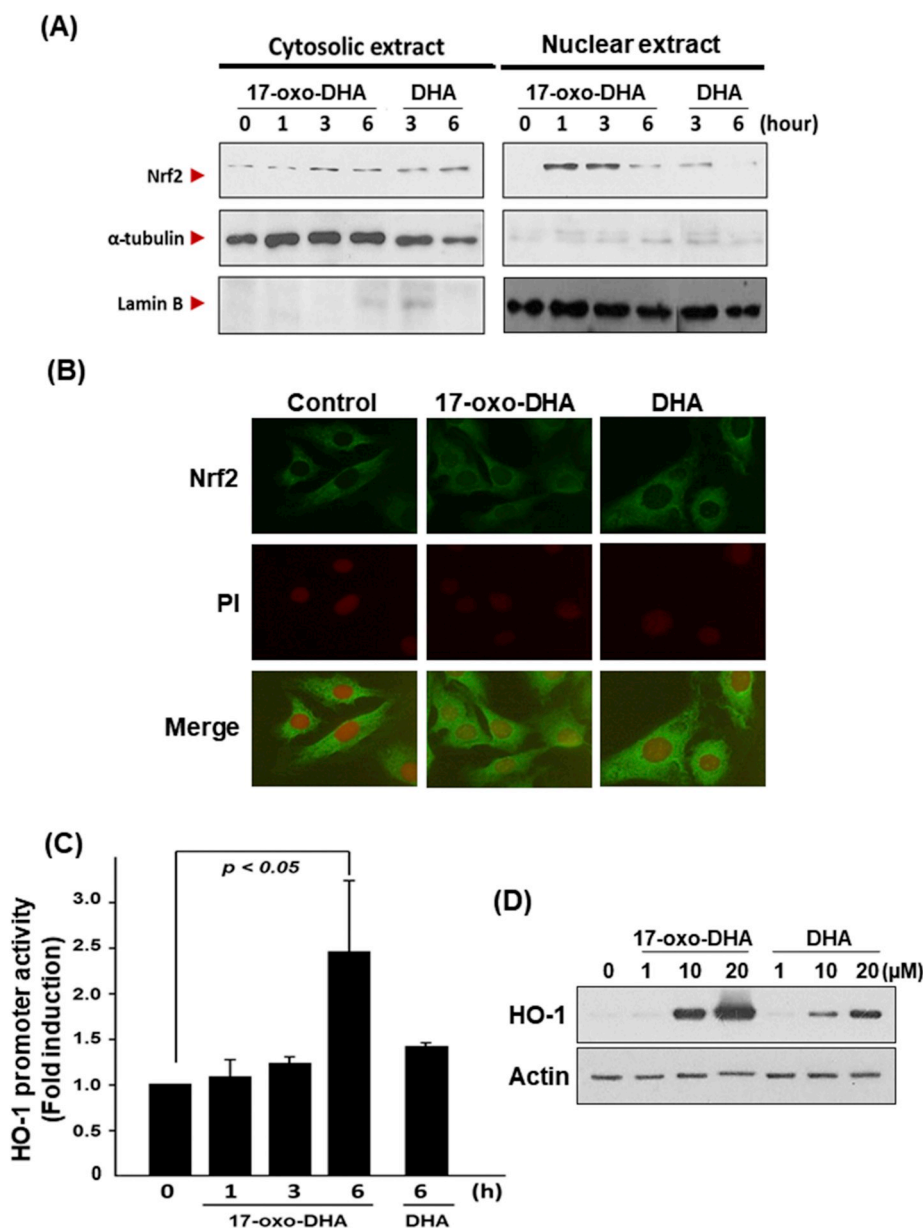
Fig. 5. Comparative effect of 17-oxo-DHA and DHA on nuclear translocation and transcriptional activity of Nrf2 and HO-1 expression. (A) JB6 cells were treated with 5 μM 17-oxo-DHA or 5 μM DHA and harvested at the indicated times. Nuclear and cytosolic fractions were isolated as described in Methods, and Nrf2 levels were assessed by Western blot analysis. (B) JB6 cells were treated with 5 μM of 17-oxo-DHA or the same concentration of DHA for 3 h. After fixation, samples were incubated with blocking agents, washed with PBST and then incubated overnight with primary antibody. After washing with PBS, samples were then incubated with FITC-conjugated secondary antibody for 1 h. Cells were also stained with propidium iodide (PI) and examined under a fluorescent microscope. (C) JB6 cells were transfected with the pHO15-luc vector for 24 h and were then treated with 5 μM 17-oxo-DHA or 5 μM DHA for indicated time periods. The cells were then lysed and analyzed for luciferase activity. Data were expressed as means of ± SD. (D) JB6 cells were treated with 1, 10, 20 μM 17-oxo-DHA or DHA for 24 h, and total proteins were isolated and subjected to the Western blot analysis for the measurement of HO-1 expression.

3.2. 17-Oxo-DHA increases nuclear translocation of Nrf2 while it decreases the steady-state level of Keap1 in hairless mouse skin

After topical application of 17-oxo-DHA (20 nmol) onto the dorsal skin of female HR-1 hairless mice, there were marked increases in nuclear accumulation of Nrf2 (Fig. 2A) with a concomitant decrease in the cytoplasmic levels of Keap1 (Fig. 2B). Like DHA, 17-oxo-DHA did not change the level of their mRNA transcripts when topically applied onto mouse skin (Fig. 2C). These data suggest that 17-oxo-DHA stabilizes Nrf2 protein, while stimulating the degradation of Keap1. The 17-oxo-DHA treatment also induced HO-1 expression (Fig. 2D) which was verified by immunofluorescence staining (Fig. 2E).

3.3. 17-Oxo-DHA induces nuclear translocation of Nrf2 and subsequent expression of its target proteins to a greater extent than DHA

In the next experiment, we compared the capability of DHA and its reactive metabolite, 17-oxo-DHA on Nrf2 activation and its target protein expression in mouse skin. Notably, there was more pronounced localization of Nrf2 in nucleus (Fig. 3A) and expression of HO-1 and NQO-1 (Fig. 3B) induced by 17-oxo-DHA, even at a lower dose (20 nmol), than that achieved with the parent compound, DHA (10 μmol). Taken together, these results suggest that 17-oxo-DHA may contribute, at least in part, to Nrf2-mediated anti-oxidative response induced by topically applied DHA in mouse skin *in vivo*.

3.4. 17-OH-DHA, a non-electrophilic metabolic precursor of 17-oxo-DHA, is a weaker activator of Nrf2-mediated antioxidant signaling

It is speculated that the electrophilic nature of 17-oxo-DHA confers this metabolite the potential to trigger the Nrf2-mediated anti-oxidative response in mouse skin. To test this supposition, the comparative effects of 17-oxo-DHA and its non-electrophilic precursor, 17-OH-DHA (Fig. 4A) on Nrf2-Keap1 signaling were examined. As shown in Fig. 4B, 17-oxo-DHA strongly induced nuclear translocation of Nrf2 and cytoplasmic degradation of Keap1, but 17-OH-DHA had little effects. Likewise, 17-OH-DHA was a much weaker inducer of the expression of HO-1 and NQO-1 in mouse skin compared with 17-oxo-DHA (Fig. 4C).
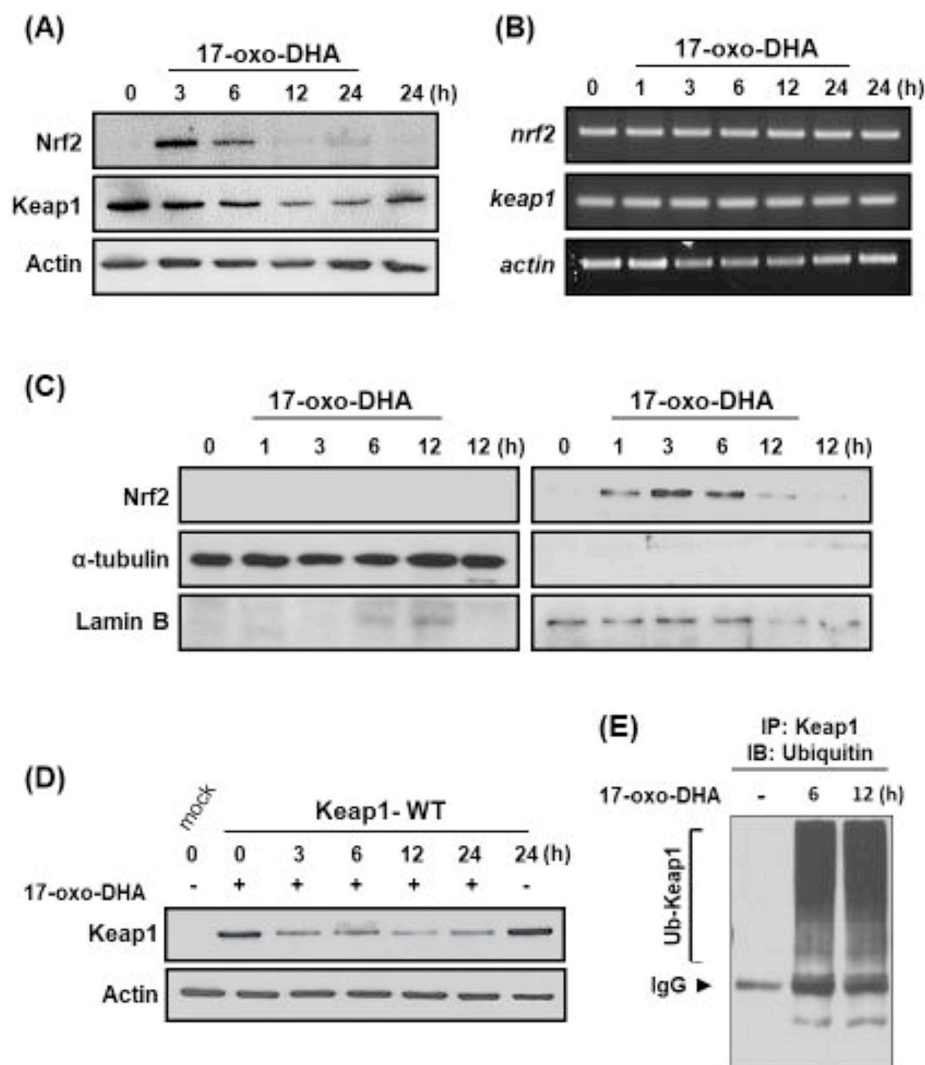
Fig. 6. 17-Oxo-DHA-induced nuclear accumulation of Nrf2 through Keap1 degradation in JB6 cells. (A, B) JB6 cells were treated with 5 μM 17-oxo-DHA for indicated time, and total protein and mRNA were isolated for measurement of Nrf2 and Keap1 by Western blot analysis (A) and RT-PCR (B), respectively. (C) JB6 cells treated with 5 μM 17-oxo-DHA were harvested at the indicated times. Nuclear and cytosolic fractions were isolated, and Nrf2 levels were analyzed by Western blot analysis. (D) HEK293T cells were transfected with wild-type Flag-Keap1 expression vector and treated with 5 μM of 17-oxo-DHA for indicated time periods. The levels of exogenous *keap1* mRNA were measured by RT-PCR. (E) JB6 cells were treated with or without 17-oxo-DHA and harvested after 6 or 12 h. Total protein was then subjected to immunoprecipitation with Keap1 primary antibody followed by immunoblotting with antibody against ubiquitin to visualize the ubiquitinated Keap1.

3.5. 17-Oxo-DHA induces nuclear translocation of Nrf2 and Nrf2-mediated HO-1 expression in cultured mouse epidermal cells

After confirming the activation of Nrf2-mediated antioxidant signaling by DHA and 17-oxo-DHA in mouse skin *in vivo*, we attempted to extend our findings to an *in vitro* model, using mouse epidermal JB6 cells. 17-Oxo-DHA (5 μM) treated to JB6 cells for 3 h increased the level of Nrf2 in the nuclear fraction whereas the same concentration of DHA barely induced nuclear accumulation of Nrf2 (Fig. 5A). 17-Oxo-DHA as well as DHA at this concentration was not cytotoxic. The immunocytochemical analysis further demonstrates that 17-oxo-DHA is more effective in inducing nuclear translocation of Nrf2 than DHA (Fig. 5B). The Keap1 degradation was also more prominent in 17-oxo-DHA-treated JB6 cells, compared with that in cells treated with DHA (data not shown).

The promoter regions of HO-1 and many other antioxidant enzymes harbour the consensus sequence, called ARE (or EpRE), a binding site for Nrf2. To investigate the 17-oxo-DHA-induced transcriptional regulation of HO-1 by Nrf2, a promoter activity assay was conducted by transfecting JB6 cells with an HO-1 promoter luciferase plasmid. As illustrated in Fig. 5C, treatment with 5 μM 17-oxo-DHA increased the luciferase activity, whereas the same concentration of DHA was not

effective. 17-Oxo-DHA also displayed a more potent effect on HO-1 protein expression than did DHA (Fig. 5D).

3.6. 17-Oxo-DHA stabilizes Nrf2 by decreasing the steady state level of Keap1 in cultured JB6 cells

Nrf2 protein expression in JB6 cells was transiently up-regulated upon treatment with 5 μM 17-oxo-DHA (Fig. 6A). In contrast to the effects on Nrf2 protein expression, the same treatment failed to alter the level of its mRNA transcript (Fig. 6B). The accumulated Nrf2 was predominantly localized in the nucleus (Fig. 6C). These results indicate that 17-oxo-DHA regulates Nrf2 expression primarily at the post-transcriptional level, rather than through *de novo* synthesis. The stability of Nrf2 is mainly regulated by Keap1, which is a substrate adaptor protein for Cul3-dependent E3 ubiquitin ligase complex. Thus, Keap1 represses Nrf2 by targeting this transcription factor for ubiquitination and subsequent proteasome-mediated degradation [14–16]. It has been reported that ROS and electrophiles can cause switching of Cul3-dependent ubiquitination of Nrf2 to Keap1, directing Keap1 to proteasomal degradation [17,18]. As 17-oxo-DHA has an electrophilic moiety, it may facilitate the Keap1 degradation through direct interaction. 17-Oxo-DHA markedly decreased the steady-state level of Keap1 protein
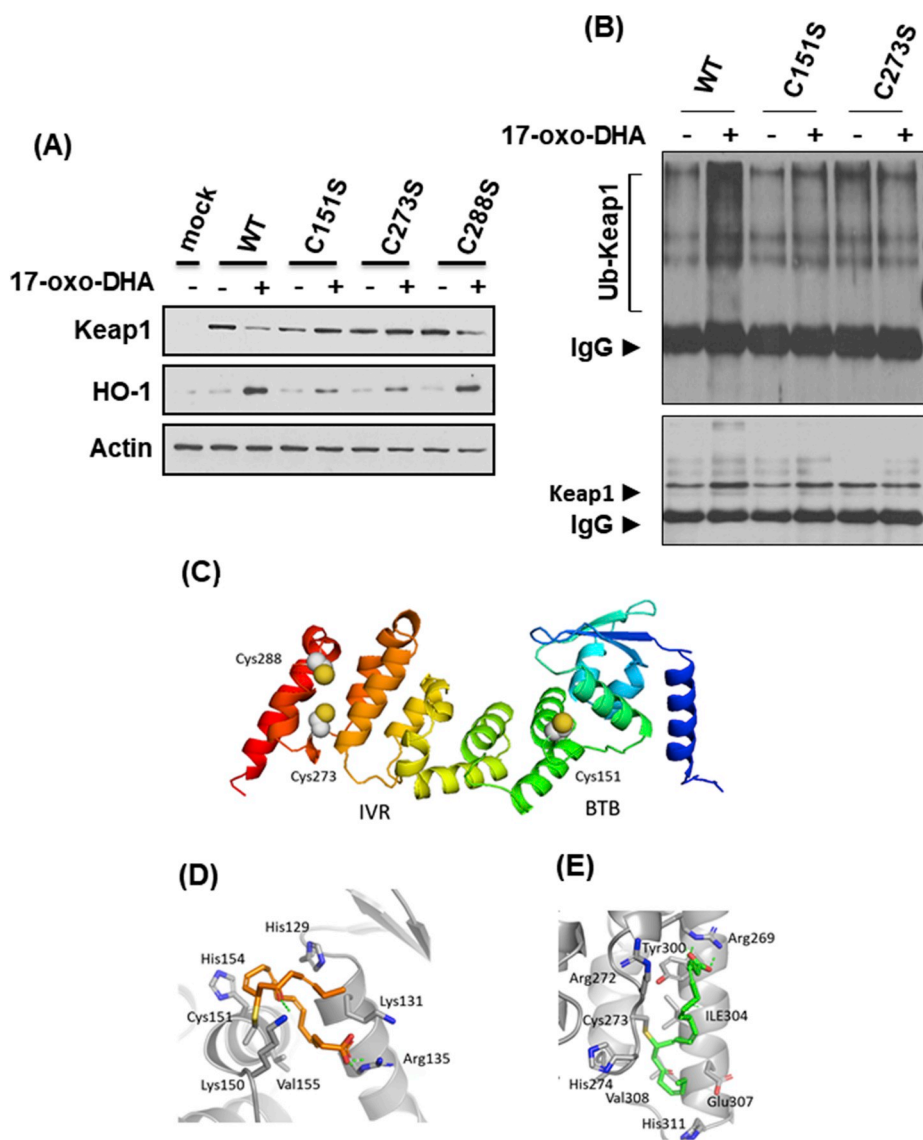
Fig. 7. Effects of C151S, C273S, and C288S mutation of Keap1 on 17-oxo-DHA mediated transactivation of HO-1 expression. (A) HEK293T cells were transfected with expression vectors for wild-type Flag-Keap1 or mutant Keap1 for 24 h. Transfected cells were treated with or without 17-oxo-DHA and harvested at 6 h. Then, the expression levels of Keap1 and HO-1 were determined by immunoblot analysis. (B) Total protein was isolated, and Keap1 ubiquitination, and expression levels of Keap1 and HO-1 were analyzed as described in the legend to Fig. 6. (C) Covalent docking pose of 17-oxo-DHA in BTB and IVR domains of mouse Keap1 protein. An *in silico* docking model of Keap1 BTB and IVR domains with three cysteine residues in the positions of 151, 273 and 288. (D, E) The covalent adduction between carbon 15 of 17-oxo-DHA and Cys151 (D) and Cys273 (E) residues is shown. Gray carbon-capped sticks represent the amino acids of Keap1. Green dashed lines are hydrogen bonds.

(Fig. 6D), corroborating the above supposition. However, its mRNA level remained constant under the same experimental conditions (Fig. 6E). Corroboratory results were also obtained with HEK 293T cells transfected with an expression vector for wild-type Flag-Keap1, whereby treatment with 17-oxo-DHA markedly reduced the steady-state level of Keap1 (Fig. 6F). To determine whether Keap1 could be ubiquitinated in response to 17-oxo-DHA treatment, JB6 cells were treated with the compound for 6 h or 12 h, lysed and subjected to immunoprecipitation with Keap1 antibody followed by immunoblotting with anti-ubiquitin antibody. As shown in Fig. 6G, 17-oxo-DHA markedly increased Keap1 ubiquitination in JB6 cells.

3.7. Effects of Keap1 cysteine thiol modification by 17-oxo-DHA in its activation of Nrf2-dependent transcription

Keap1 is a cysteine rich protein, with 27 cysteine residues in the human and 25 in the murine protein. It has been reported that some critical sensor cysteines of Keap1 can be oxidized by ROS or covalently modified by electrophiles, leading to its conformational changes. This disrupts the interaction between Keap1 and Nrf2, thereby preventing degradation of newly synthesized Nrf2 [32]. As 17-oxo-DHA harbours an α,β-unsaturated carbonyl moiety, it can serve as an electrophile toward cellular nucleophiles, including cysteine thiols of Keap1. Several

studies have identified 3 critical cysteine residues, Cys151, 273, and 288, present in Keap1 as sensors for electrophiles and oxidants that are speculated to be involved in regulating the activity of Nrf2 [32].

In order to determine which of these cysteine residues is/are involved in the activation of Nrf2 following 17-oxo-DHA treatment, we utilized expression vectors for Keap1 mutants in which each of aforementioned 3 sensor cysteine residues was replaced by serine. HEK293T cells transfected with expression vectors for wild-type, Keap1-C151S, Keap1-C273S, or Keap1-C288S were treated with or without 17-oxo-DHA, and the lysates were subjected to immunoblot analysis with antibodies against Keap1 and HO-1. The Keap1 degradation and HO-1 induction observed in the wild-type cells were abrogated in cells harbouring Keap1 C151S or C273S mutation, but not in cells harbouring C288S (Fig. 7A). Next, we attempted to find out whether these cysteine residues are also involved in the 17-oxo-DHA-induced ubiquitination of Keap1. As seen in Fig. 7B, a mutation at Cys151 or Cys273 abolished ubiquitination of Keap1, suggesting that these cysteine residues are also required for 17-oxo-DHA-induced proteasomal degradation of Keap1.

As an initial step toward determining whether Cys151 and Cys273 are putative sites for 17-oxo-DHA binding to Keap1, prediction of covalent docking pose of 17-oxo-DHA the mouse Keap1 homology model was performed. Cys151 is located at the tip of flexible loop in BTB domain, while Cys273 is in the intervening region of IVR domain (Fig. 7C). *In*
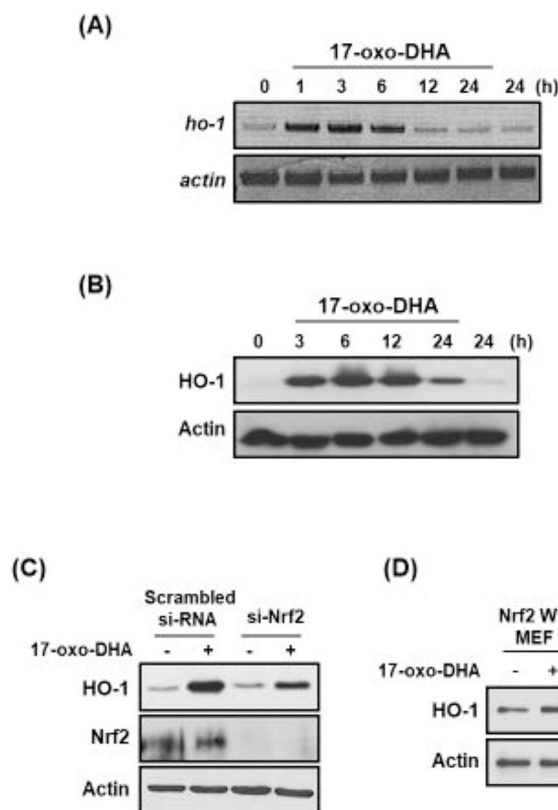
Fig. 8. Role of Nrf2 in 17-oxo-DHA induced upregulation of HO-1. (A, B) JB6 cells were treated with 5 μM 17-oxo-DHA for indicated time, and the expression levels of HO-1 mRNA (A) and protein (B) levels were measured by RT-PCR and Western blot analysis, respectively. (C) JB6 cells were transiently transfected with 20 nM of si-Nrf2 for 24 h, and 17-oxo-DHA-induced HO-1 expression was determined by Western blot analysis. (D) Nrf2-WT or Nrf2-Knockout MEF cells were incubated with or without 5 μM 17-oxo-DHA and harvested after 6 h. Total protein was isolated, and the HO-1 expression level was measured by Western blot analysis.

silico docking analysis reveals that the sulfur atom of both cysteine residues is likely to form a covalent bond with the electrophilic sp^2 carbon 15 of 17-oxo-DHA through Michael addition. Carbonyl and carboxyl groups of 17-oxo-DHA adducted to Cys151 were predicted to form hydrogen bonds with Lys150 and Arg135 residues, and a hydrophobic chain is positioned at positively charged residues His129, Lys150 and His154 (Fig. 7D). 17-Oxo-DHA covalently bound to Cys273 is predicted to interact via a hydrogen bond with Arg269 at the narrow binding pocket localized between helix bundles of the IVR domain (Fig. 7E).

3.8. 17-Oxo-DHA induces upregulation of HO-1 expression through Nrf2 activation

In parallel with accumulation of Nrf2, both transcription (Fig. 8A) and translation (Fig. 8B) of HO-1 were elevated by 17-oxo-DHA in a time-dependent manner. In a subsequent experiment, Nrf2 was silenced by transfecting JB6 cells with siRNA, and the expression of HO-1 following 17-oxo-DHA treatment was measured by Western blot analysis. As compared to cells transfected with scrambled siRNA, 17-oxo-DHA-induced expression of HO-1 was abrogated in cells transfected with siNrf2 (Fig. 8C), indicating that Nrf2 does mediate 17-oxo-DHA-induced upregulation of HO-1 expression in mouse epidermal cells. Furthermore, we utilized embryonic fibroblasts derived from Nrf2 wild-type and knockout mice (MEFs) to further verify the role of Nrf2 in HO-1 upregulation by 17-oxo-DHA. 17-Oxo-DHA induced the expression of HO-1 in Nrf2 wild-type MEFs, while this was blunted in Nrf2 knockout MEFs (Fig. 8D).
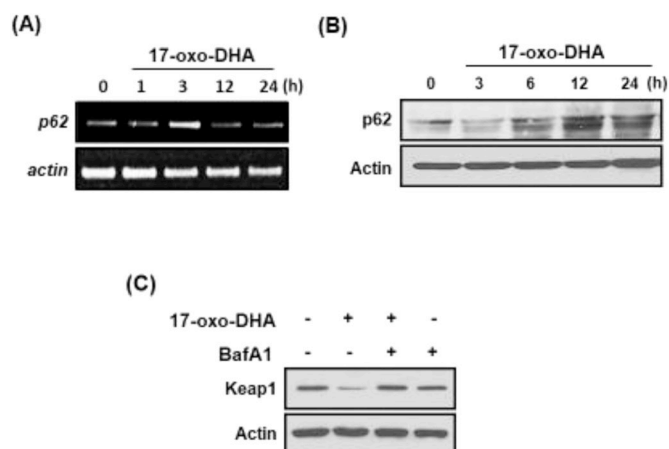
Fig. 9. 17-Oxo-DHA-induced degradation of Keap1 through p62-mediated autophagy. (A, B) JB6 cells were treated with 5 μM 17-oxo-DHA for indicated time, and total mRNA (A) and protein (B) were isolated, and the p62 mRNA and protein expression were measured by RT-PCR and Western blot analysis, respectively. (C) Effects of an autophagy inhibitor, bafilomycin on Keap1 protein accumulation in JB6 cells.

3.9. 17-Oxo-DHA induces degradation of Keap1 through p62-mediated autophagy

Nrf2 is also regulated by p62 (also known as SQSMT1; sequestosome), the ubiquitin-binding protein that serves as an autophagy substrate and a cargo receptor. Besides direct modification of Keap1 by oxidants or electrophiles, its interaction with p62 promotes dissociation of Nrf2 from Keap1 [33]. Based on our finding that 17-oxo-DHA induces ubiquitination of Keap1, we tested the possible involvement of p62 in degradation of ubiquitin-conjugated Keap1 following 17-oxo-DHA treatment. We observed a time-dependent increase in *p6*2 mRNA (Fig. 9A) and p62 protein (Fig. 9B) levels upon 17-oxo-DHA treatment, which coincided with the gradual decrease in the Keap1 protein level (Fig. 6D). To further determine whether 17-oxo-DHA-induced degradation of Keap1 is associated with p62-mediated autophagy, we examined the Keap1 protein accumulation in JB6 cells treated with 17-oxo-DHA in the presence of an autophagy inhibitor, bafilomycin. Bafilomycin restored the Keap1 protein level reduced by 17-oxo-DHA (Fig. 9C).

4. Discussion

Nrf2 is a redox-sensitive master regulator of genes encoding many cytoprotective enzymes required for cellular defence against oxidative stress and other noxious conditions [10,11,34]. Among the enzymes involved in Nrf2-mediated cellular stress response, HO-1 is of particular interest because its expression is commonly induced by a wide array of environmental and endogenous stressors. In various cells, the induction of HO-1 confers protection against oxidative stress [35,36]. Dysfunction of the aforementioned cytoprotective mechanism in the skin has been associated with severe dermal stress and photocarcinogenesis [37,38]. In line with this notion, Nrf2 knockout mice were shown to be more susceptible to chemically induced skin carcinogenesis [39].

A vast variety of anti-oxidative agents, many of which are constituents of our daily diet, have been demonstrated to combat oxidative skin damage and photocarcinogenesis [40]. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) abundant in fish oil have multiple health beneficial effects, which are attributable to their anti-oxidative and anti-inflammatory activities [23–27,41]. DHA, a representative n-3 PUFA, modulates activities of redox-sensitive pro-inflammatory transcription factors, such as NFκB [42,43] and STAT3 [44]. Our recent studies have demonstrated that topically applied DHA inhibits UVB-induced

carcinogenesis in mouse skin [44].

In view of the protective role of DHA in skin disorders often caused by UV-induced oxidative tissue damage, we sought to first investigate whether topical application of DHA could provoke anti-oxidative response in mouse skin *in vivo*. We noticed that topically applied DHA increased nuclear translocation of Nrf2, thereby upregulating anti-oxidant enzyme expression. Recent studies have revealed the distinct set of novel endogenous lipid mediators, generated from n-3 PUFAs either enzymatically or through non-enzymatic oxidation reactions, exert health beneficial effects. Among them, EFOXs are of principal interest because of their potential to modulate broad range of signaling molecules [28]. By forming reversible adducts with nucleophiles through Michael addition, these electrophilic species modulate multiple redox-sensitive signaling pathways including those mediated by Keap1/Nrf2.

Electrophilic compounds bind Keap1 and disrupt proteasome-mediated degradation of Nrf2. 17-Oxo-DHA that has an α,β-unsaturated ketone moiety capable of directly binding Keap1 and activating Nrf2 in cultured macrophages [28,29,45]. In our present study, 17-oxo-DHA was found to be a more potent mediator than the non-electrophilic precursor DHA in inducing the Nrf2-driven expression of cytoprotective enzymes. Thus, 17-oxo-DHA, at a dose as low as 20 nmol, activated Nrf2-mediated anti-oxidant signaling response to a greater extent than DHA. 17-Oxo-DHA not only enhanced the steady-state level of Nrf2 at post-translational levels, but also reduced the stability of Keap1 through ubiquitin-proteasomal degradation. In contrast to 17-oxo-DHA, its non-electrophilic metabolic precursor 17-OH-DHA induced much weaker induction of Nrf2 activation and its target protein expression. 17-OH-DHA may undergo oxidation to 17-oxo-DHA when topically treated to mouse skin, possibly by epidermal dehydrogenase activity, and this accounts for its marginal induction of Nrf2-HO-1 signaling.

Recently, extensive studies have been conducted to elucidate the mechanism whereby cysteine modifications in Keap1 lead to Nrf2 activation. Although there may exist several different possibilities of Nrf2 activation, the proposed mechanisms share one important perspective: Keap1 is not just a repressor that sequesters Nrf2 in the cytoplasm, but

rather acts as an active mediator that targets Nrf2 for ubiquitination and proteasomal degradation. Thus, Keap1 functions as a substrate adaptor protein for the Cul3-dependent E3 ubiquitin ligase complex [14–16]. Keap1 is rich in cysteine residues prone to be modified by oxidants and electrophiles, and this hampers Keap1-directed ubiquitination of Nrf2 [32,46]. This prompted us to postulate that 17-oxo-DHA may interact with particular cysteine residue(s) of Keap1 and thereby causes its conformational changes, resulting in Nrf2 stabilization. The site-directed mutation studies have identified 3 critical cysteine residues (Cys151, Cys273, and Cys288) of Keap 1 that are important sensors for Nrf2 activators [32,46]. In particular, mutation of Cys151 to serine has been shown to abolish electrophile- and oxidant-mediated Nrf2 ubiquitination [46]. In line with this observation, our results indicate that Cys151 is required for stabilization of Nrf2 in response to 17-oxo-DHA treatment. Two other cysteine residues, Cys273 and Cys288 are required for Keap1 to repress Nrf2-dependent transcriptional activation. However, 17-oxo-DHA-induced Keap1 degradation was impaired in cells harbouring mutant Keap1-C273S, but not Keap1-C288S. Based on these findings, we speculate that Cys151 and Cys273 may be putative binding sites of 17-oxo-DHA.

We suggest two possible mechanisms for 17-oxo-DHA-induced activation of Nrf2 in murine epidermal JB6 cells as well as in mouse skin. One is that 17-oxo-DHA modifies Cys151 of Keap1, and dissociation of Keap1 from Cul3 abrogates Keap1 directed ubiquitination of Nrf2, thereby activating Nrf2-dependent transcription of HO-1. Moreover, it has been suggested that Cys273 may be required for binding of Keap1 to either E2 ubiquitin conjugating enzyme or E3 ubiquitin ligase complex and that Cys273 may also be involved in ubiquitin transfer from E2 enzyme to Nrf2 [32]. On the basis of these findings and our results, we suggest a second possible mechanism: 17-oxo-DHA modifies the Cys273 of Keap1, and thereby inhibiting Keap1-mediated ubiquitination of Nrf2, resulting in Nrf2 stabilization. Though Cys151 and Cys273 have been predicted to be potential sites to which 17-oxo-DHA binds, still more direct evidence can be attained by mass-spectral analysis of digested peptides derived from modified Keap1.

Still another plausible mechanism by which 17-oxo-DHA induces
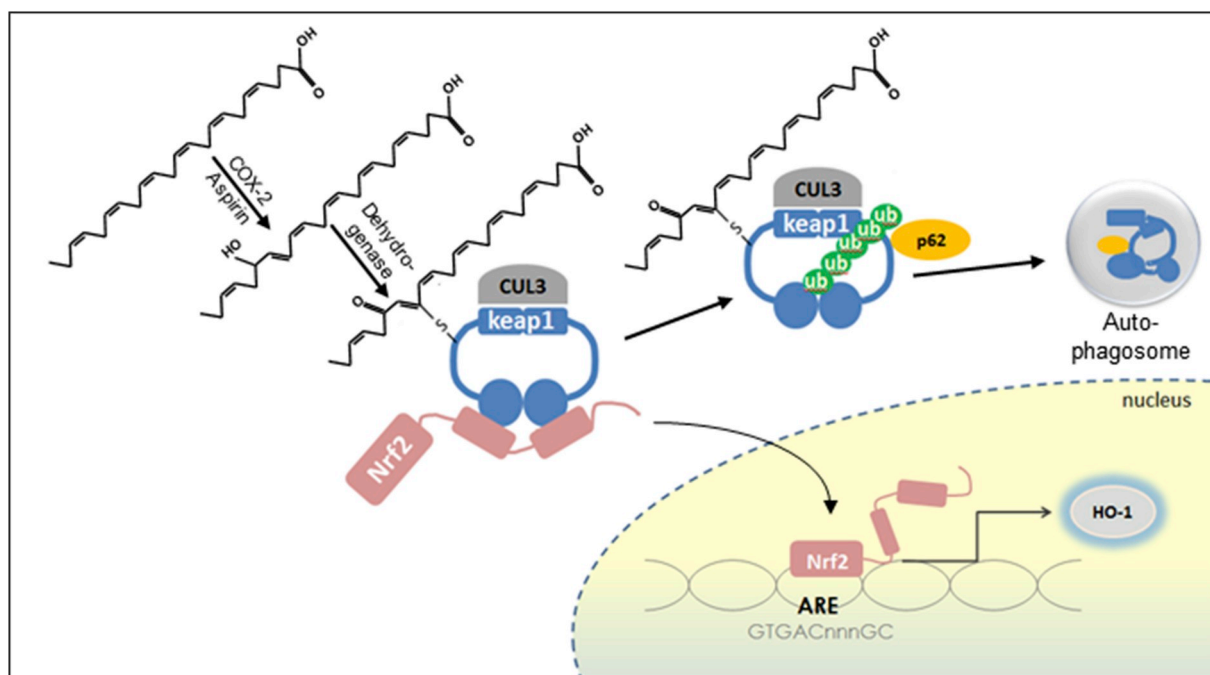
Fig. 10. Schematic representation of Nrf2 activation and antioxidant gene expression by 17-oxo-DHA. 17-Oxo-DHA, an electrophilic oxo-derivative of DHA, harbours an α,β-unsaturated carbonyl moiety, which is reactive towards cellular nucleophiles, such as cysteine residues of Keap1. Modification of specific thiol residues of Keap1 leads to disruption of the Keap1-Nrf2 complex formation. This allows Nrf2 to translocate into the nucleus, where it binds to the ARE and activates transcription of target genes (e.g., HO-1). Keap1 modification by 17-oxo-DHA also facilitates Keap1 degradation through p62-mediated autophagy.

Nrf2 activation involves the autophagic degradation of its inhibitory protein, Keap1. It has been reported that Keap1 ubiquitinated by a Cul3-dependent complex undergoes proteasome-independent degradation [18]. p62, a scaffold protein, binds ubiquitin-positive cargos, and targets them for degradation via autophage [47]. In agreement with this notion, Keap1 degradation was found to be partly associated with p62-mediated autophagy [33].

Several studies have suggested that p62 provokes the stabilization of Nrf2 by directly interacting with Keap1, which results in disruption of the Keap1-Nrf2 complex [33,48,49]. It has been demonstrated that certain electrophiles accelerate the degradation of covalently modified Keap1 by autophagy [50]. Likewise, it is conceivable that a conformational change of Keap1 induced by electrophilic 17-oxo-DHA may facilitate p62-mediated autophagic degradation of Keap1 protein. How structural modification of Keap1 by 17-oxo-DHA affects its interaction with p62 for degradation via autophage merits further investigation. Phosphorylation of p62 at specific serine residues enhances its affinity for Keap1 as well as cargo protein. It'll be worthwhile determining whether 17-oxo-DHA can promote phosphorylation of p62, thereby facilitating the recruitment of Keap1 to the ubiquitinated cargo.

In conclusion, 17-oxo-DHA induces an Nrf2-mediated antioxidant response in mouse skin *in vivo* and cultured murine epidermal cells, which is attributable to its direct interaction with some sensor cysteine residues of Keap1 (Fig. 10). We speculate that 17-oxo-DHA-induced modification of Cys151 or Cys273 leads to activation of Nrf2 by inhibiting Keap1 dependent ubiquitination of Nrf2. Alternatively, 17-oxo-DHA may promote p62-mediated autophagic degradation of Keap1. Thus, 17-oxo-DHA holds great promise for use in the management of skin diseases characterized by persistent oxidative stress or inflammation.

Declaration of competing interests

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.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2019.108156.

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