약학석사학위논문

17-Oxo-DHA Induces Heme Oxygenase-1 Expression in Mouse Epidermal JB6 Cells through Nrf2 Activation

마우스 피부 상피세포에서 17-oxo-DHA 에 의해
유도된 Nrf2 의 Heme Oxygenase-1 발현 효과

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Abstract

17-Oxo-DHA Induces Heme Oxygenase-1 Expression in Mouse Epidermal JB6 Cells through Nrf2 Activation

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Recently, growing attention has been given to the discovery of new classes of bioactive mediators derived from omega-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and to their role as endogenous anti-inflammatory signaling mediators. 17-Oxo-DHA is an electrophilic metabolite of DHA, generated by the action of cyclooxygenase-2 (COX-2) and a dehydrogenase. Owing to its electrophilic nature, 17-oxo-DHA modulates multiple redox-sensitive signaling mechanisms including Nrf2-induced anti-oxidant response. In the present study, the molecular mechanism responsible for activation of Nrf2-Keap1 signaling in response to 17-oxo-DHA treatment was investigated in mouse epidermal JB6 cells. 17-Oxo-DHA significantly enhanced nuclear translocation and transcriptional activity of Nrf2 and its target protein heme oxygenase-1 (HO-1). Notably, 17-oxo-DHA was a more potent Nrf2 activator than its parent molecule, DHA. Knockdown of Nrf2 in JB6 cells diminished the 17-oxo-DHA induced upregulation of HO-1. Similar results were obtained with experiments using embryofibroblasts from Nrf2 knockout mice, corroborating that Nrf2 is essential for 17-oxo-DHA induced upregulation of HO-1 expression. 17-Oxo-DHA also markedly reduced the expression of Keap1 protein in posttranslational levels by inducing ubiquitination of Keap1. In identifying a critical Keap1 cysteine residue as a potential target of 17-oxo-DHA, I showed that mutation of Cys151 in Keap1 not only abolished the 17-oxo-DHA induced expression of HO-1, but also 17-oxo-DHA-induced ubiquitination of Keap1, suggesting that 17-oxo-DHA may interact with this cysteine residue

directly. Interestingly, Keap1 degradation in response to 17-oxo-DHA coincided with p62 accumulation. 17-oxo-DHA-induced Keap1 degradation was abolished by an autophagy inhibitor, suggesting that Keap1 degradation is associated with p62-mediated autophagy. Treatment with 17-oxo-DHA generated high molecular weight (HMW) form of Keap1 in HEK293 cells harboring wild-type FLAG-Keap1, whereas in cells harboring C151S mutant form of Keap1, HMW forms were not observed. Taken together, Cys151 residue of Keap1 appears to be a critical target of 17-oxo-DHA in its induction of antioxidant expression and Keap1 degradation. Our results provide insight into 17-oxo-DHA as a novel and potent endogenous bioactive lipid mediator involved in anti-oxidant response.

Keywords:

17-oxo-DHA, JB6, Heme Oxygenase-1, Nrf2, Keap1, Autophagy, p62

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Introduction

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) and cellular antioxidant capacity. Mounting evidence in both experimental and clinical studies suggests that oxidative stress is implicated in diverse pathological conditions such as obesity[12], Diabetes Mellitus[20], neurodegenerative disorders[31], and several types of cancers[26]. High levels of ROS with inadequate stress response cause oxidative stress that leads to damage of important biomolecules like DNA, which contributes to mutagenesis and carcinogenesis[22]. It is therefore important to identify molecules that can counteract the detrimental effect of ROS and prevent the disruption of the redox homeostasis by modulating the expression of antioxidant genes.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive master regulator involved the induction of the detoxifying enzymes and antioxidant proteins, which plays key role in cellular defense against oxidative and xenobiotic stress[21]. Among the cytoprotective proteins involved in cellular stress response, Heme oxygenase-1 (HO-1) is of particular interest because its expression is commonly induced by a wide array of noxious stimuli. HO-1 plays an essential role in protecting cells against oxidative stress and in re-establishment of homeostasis by its antioxidant and anti-inflammatory actions[28]. Therefore, induction of cytoprotective enzymes by inducers through Nrf2 pathway represents an important part of cancer preventive strategy.

Chemoprevention represents a promising strategy in which natural or synthetic agents are used to inhibit, reverse or retard tumorigenesis[29]. In particular, the protective effects of Docosahexaenoic acid (DHA) against pathogenesis of some cancers have been evidenced in the growing body of epidemiological[19, 23], clinical and experimental studies[24]. Recent studies on the formation of bioactive metabolites derived from ω-3 polyunsaturated fatty acids (PUFA) have revealed the distinct set of novel endogenous lipid mediators that can stimulate the molecular events that can promote chemopreventive effect. Several bioactive oxygenated ω-3 fatty acids are generated by either enzymatic or non-enzymatic oxidation reactions. Among the oxygenated metabolites of ω-3 fatty acids, electrophilic species are of prime interest because of their potential to modulate broad range of signaling pathways by interacting with cellular nucleophiles[8]. By forming adducts with nucleophiles

through Michael addition reaction, electrophiles modulate multiple redox-sensitive signaling pathways such as Keap1/Nrf2 pathway.

17-Oxo-DHA is a recently discovered endogenous electrophilic α,β-unsaturated ketone derivative of DHA which is generated by the action of cycloxygenase-2 (COX-2) and a dehydrogenase[8]. Reported findings show that owing to its electrophilic nature, 17-oxo-DHA exerts anti-oxidant and anti-inflammatory actions via Nrf2-dependent mechanisms. However, the underlying cellular and molecular mechanism has not been fully elucidated.

Further mechanistic studies on the molecular mechanism governing the actions of 17-oxo-DHA are required to facilitate the evaluation of its chemopreventive potential. In the present study, I investigated the molecular mechanism of the Nrf2-mediated induction of cytoprotective enzymes by 17-oxo-DHA in mouse epidermal JB6 cells. In comparison with the non-electrophilic precursor DHA, 17-oxo-DHA was shown to be a more potent mediator in inducing expression of cytoprotective enzymes, suggesting that this electrophilic derivative of DHA may represent an ultimate bioactive lipid mediator that contributes to the previously observed beneficial physiological actions of DHA.

Materials and Methods

Chemicals

17-oxo-DHA (17-keto-4(Z),7(Z),10(Z),13(Z),15(E),19(Z)-Docosahexaenoic Acid) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Eagle's Minimum Essential Media (MEM), fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). Dithiothreitol (DTT) were purchased from Sigma Chemical Co.(St. Louis, MO, USA). Rabbit polyclonal HO-1 antibody was a product of Stressgen (Ann Arbor, MI, USA). Primary antibodies for Nrf2 and Keap1 were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies for NQO1 were supplied by Abcam (Cambridge, UK). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). The enhanced chemiluminescent detection reagent was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL, USA). The Nrf2 siRNA (5'-UUCUGUCGCUGACUAAAGUCAAACA-3') and Stealth^{TM} universal RNAi negative control duplexes were purchased from Invitrogen (Carlsbad, CA, USA). Bicichoninic acid (BCA) protein assay reagent was a product of Pierce Biotechnology (Rockfold, IL, USA). Polyvinylidene difluoride (PVDF) membrane was supplied from Gelman Laboratory (Ann Arbor, MI, USA).

Cell culture

Mouse epidermal JB6 Cl 41 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in monolayers in MEM, containing 5 % FBS, 1.5 mg/mL sodium bicarbonate. Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO2 and 95 % air. MEF cells were cultured in DMEM supplemented with 10 % FBS and 1 % of antibiotic-antimucotic mixture and kept at 37 °C, 5 % CO_2.

Preparation and maintenance of mouse embryonic fiboblasts (MEF)

The *Nrf2* wild type (*Nrf2*$^{+/+}$) and *Nrf2*-null (*Nrf2*$^{-/-}$) mice were originally provided by Dr. Jeffery Johnson, University of Wisconsin, Madison, USA. After in-house breeding, the *Nrf2*$^{-/-}$, *Nrf2*$^{+/-}$ and wild-type mice were maintained in the animal quarters and were housed in a 12-h light/dark cycle, The were fed standard rodent chow and given water ad libitum. Male and female *Nrf*$^{+/-}$ were mated, and embryos were obtained at the day 13.5 after pairing under aseptic conditions. The heads of the embryos were used to confirm the *Nrf2* genotype by polymerase chain reaction, and the embryo

bodies were minced into small pieces and cultured in high glucse DMEM supplemented with 10 % FBS and kept at 37 °C with 5 % CO_2

Transient transfection of small interfering RNA (siRNA) and expression vectors

Cells at a confluence of 60-70% were transfected with control siRNA or Nrf2 siRNA (20 μM) using Lipofectamine® RNAi-MAX reagent (Invitrogen) according to the manufacturer's instructions. After 36 h of transfection, cells were treated with 17-oxo-DHA for additional 6 h, and cell lysates were prepared. The target sequence for Nrf2 siRNA is as follows: forward 5'-AAG AGU AUG AGC UGG AAA AAC UU-3' and reverse 5'-GUU UUU CCA GCU CAU ACU CUU UU-3'. Keap1 expression vectors (pCMV-Flag-Keap1) were transfected to HEK293T cells using Lipofectamine® 2000 reagent according to manufacturer's instructions.

Western blot analysis

Preparation of cell lysate- Cells were washed with cold 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 13000xg 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 (pH7.9), 10 mM KCl, 0.3 mM EDTA, 0.2 mM DTT, 0.3 mM PMSF, 0.1 mM phenylmethylsulfonylfluoride (PMSF)], incubated for 10 min on ice and then centrifuged at 8000 rpm for 5 min at 4°C. Supernatant was collected as cytosolic extract. The residual pellets were washed with hypotonic buffer and resuspended in hypertonic buffer C [20 mM HEPES (pH7.8), 25% glycerol, 400mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF], keeping on ice for 30 min during rocking followed by centrifugation at 8000 rpm 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.

SDS-PAGE- Protein samples from whole cell lysate, cytosolic extract or nuclear fraction were mixed with sodium dodecyl sulfate (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 (Tris-based saline containing 0.1% Tween-20) 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 peroxidase-conjugated secondary antibodies in 3% fat-free dry milk-TBST for 1 h at room

temperature. The blots were rinsed again three times with PBST, and the transferred proteins were incubated with the ECL substrate detection reagent for 1 min according to the manufacturer's instructions and visualized with X-ray film in the dark room.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from JB6 Cl 41 cells by using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. 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 microliter of cDNA was amplified in sequential reactions using HS Prime Taq Premix (Genetbio). The mRNA expression of HO-1 (25 cycles of 94 °C for 30 sec, 53 °C for 30 sec, and 72 °C for 30 sec), NQO1 (25 cycles of 94 °C for 30 sec, 53 °C for 30 sec, and 72 °C for 30 sec), Actin (20 cycles of 94 °C for 30 sec, 52 °C for 30 sec, and 72 °C for 30 sec), Nrf2 (25 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec), Keap1 (23 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec), were determined. These PCR cycles were followed by final extension for 7 min at 72 °C. The primers used for each RT-PCR reactions are as follows (forward and reverse, respectively): *HO-1,* 5'-TAC ACA TCC AAG CCG AGA AT-3' and 5'-GTT CCT CTG TCA GCA TCA CC-3'; *NQO1,* 5'-TCG GAG AAC TTT CAG TAC CC-3' and 5'-TGC AGA GAG TAC ATG GAG CC-3'; *KEAP1,* 5'-GGC AGG ACC AGT TGA ACA GT -3' and 5'-GGG TCA CCT CAC TCC AGG TA-3' and *Actin,* 5'-AGA GCA TAG CCC TCG TAG AT-3' and 5'-CCC AGA GCA AGA GAG GTA TC-3'. Amplification products were resolved by 2.0 % agarose gel electrophoresis, stained with SYBR green, and photographed using fluorescence in LAS-4000.

HO-1 promoter luciferase reporter gene assay

JB6 Cl 41 cells were cultured up to 50 % confluence in 6-well plates in complete media. Cells were then transfected with with pHO15-luc vector (1 μg) using Lipofectamine® 2000 for 24 h. After transfection, cells were treated with 17-oxo-DHA or DHA, and cell lysate was prepared by using reporter lysis buffer (Promega, Madison, WI, USA). After incubating cell lysates with a luciferase substrate, the luciferase activity was measured 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.

Immunocytochemical analysis

JB6 Cl41 cells were plated on the 4-well chamber slides at a density of 1 X 10^4 per well, 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 overnight at 4 °C with fresh blocking buffer (0.1 % Tween 20 in PBS, pH 7.4, containing 5 % bovine serum albumin) and probed with primary antibody for Nrf2 (diluted at 1:100 in 5 % BSA in PBST buffer) overnight at 4 °C. Cells were washed three times with PBST, and were incubated with fluoresceinisothiocyanate(FITC)-goat anti-rabbit IgG secondary antibody diluted in 5 % BSA in PBST buffer for 1 h at room temperature. Cells were rinsed with PBST, and cells were examined under fluorescence microscope.

Docking study

AutoDock Vina program[32](The Scripps Research Institute, CA, USA) was used to make docking model of 17-oxo-DHA into BTB domain of KEAP1. The structures of KEAP1's BTB domain are from Protein Data Bank (PDB ID: 4CXT and 4CXI). 17-oxo-DHA coordinates was drawn with the GlycoBioChem PRODRG2 Server[27] (http://davapc1.bioch.dundee.ac.uk/prodrg/). The grid maps for the docking model were set to 65 X 56 X 49 points with 1.0 Å spacing to cover all surface of BTB domain of KEAP1. The other parameters were set in default value in AutoDock Vina program.

Statistical analysis

When necessary, data were expressed as means of ±SD of at least three independent experiments, and statistical analysis for single comparison was performed using the Student's t test. The criterion for statistical significance was $p < 0.05$

RESULTS

17-Oxo-DHA is a more potent inducer of HO-1 expression than DHA.

In order to assess the potential role of 17-oxo-DHA in inducing Nrf2-mediated antioxidant response, JB6 cells were treated with 17-oxo-DHA at a concentration range of 1-10 μM for 24 h and the expression level of the target proteins were analyzed by Western blot analysis (Fig. 2A). HO-1 protein expression was markedly increased in response to 17-oxo-DHA in a concentration dependent manner, whereas the NQO1 protein expression level subsided upon 10 μM treatment. DHA, which is a non-electrophilic precursor of 17-oxo-DHA, was reported to induce Nrf2-mediated HO-1 expression[33]. To compare their effects on the expression of antioxidant enzymes in JB6 cells, expression levels of HO-1 and NQO1 protein were compared between cells treated with 17-oxo-DHA and those treated with DHA at the same concentration (Fig 2B and C). The results indicated that although both lipids elicit similar response, 17-oxo-DHA displayed a more potent effect in inducing HO-1 protein expression than DHA. In contrast, NQO1 protein expression was elevated in cells treated with DHA to a greater extent than those treated with 17-oxo-DHA. The concentration of 5 μM 17-oxo-DHA was determined based on the MTT cytotoxicity assay as the one giving the observable biological response with minimal toxicity (Fig 2D).

17-Oxo-DHA induces upregulation of Nrf2 by stabilizing the protein at post-transcriptional level and increases the mRNA expression of antioxidant enzymes.

As Nrf2 is known to play a key role in the regulation of many antioxidant and cytoprotective proteins including HO-1 and NQO1, the expression levels of Nrf2 mRNA (Fig. 3A) and protein (Fig 3.B) in the 17-oxo-DHA treated JB6 cells were examined. Nrf2 protein expression was upregulated upon treatment of 5 μM of 17-oxo-DHA, which peaked at 3 hours. In contrast to the effects on protein expression, 17-oxo-DHA treatment did not alter the steady state level of Nrf2 mRNA. These results suggest that 17-oxo-DHA regulates Nrf2 expression primarily by post-transcriptional mechanisms, rather than via *de-novo* synthesis. In order to analyze the time course in the expression of mRNA and protein, JB6 cells were treated with 17-oxo-DHA for indicated time, and mRNA and protein expression levels were analyzed by RT-PCR (Fig. 3C) and Western blot analysis (Fig 3.D),

respectively. Both HO-1 and NQO1 mRNA and protein expression were elevated by 17-oxo-DHA in time dependent manners, each reaching its maximal level at different time points. Here, the results demonstrated that 17-oxo-DHA-induced expression of HO-1 protein precedes NQO1 protein expression.

17-Oxo-DHA induces nuclear translocation of Nrf2 and induces ARE-mediated HO-1 gene expression.

The nuclear accumulation of Nrf2 is usually considered an indication of Nrf2 activation in response to stressors[14, 17]. Nuclear localization of Nrf2 in 17-oxo-DHA treated cells was examined by assessing Nrf2 levels in nuclear fraction by Western blot analysis (Fig. 4B). To further confirm that 17-oxo-DHA is a more potent inducer of Nrf2-mediated antioxidant expression than DHA, an equal concentration of DHA was treated to JB6 cells and the results were compared with those obtained from cells treated with 17-oxo-DHA. As expected, 17-oxo-DHA markedly increased the level of Nrf2 in the nuclear fraction whereas only a marginal localization of nuclear Nrf2 was obtained from DHA treated JB6 cells. In particular, Nrf2 accumulation peaked between 1-3 h following 17-oxo DHA treatment, which was in corroboration with the result obtained with the whole-cell lysates. As shown in Figure 4A, Nrf2 was translocated into the nucleus following 17-oxo-DHA treatment. This immunocytochemical analysis of 17-oxo-DHA treated JB6 cells was also conducted in comparison with the DHA treated cells to further demonstrate that 17-oxo-DHA is more effective in inducing nuclear translocation than DHA (Fig. 4A).

A group of cytoprotective enzymes, including HO-1, is believed to be under the transcriptional control of antioxidant response elements (ARE), a binding site for transcription factor Nrf2[10]. To investigate whether 17-oxo-DHA-induced upregulation of HO-1 is mediated by transcriptional activation, a promoter activity assay on *ho-1* was conducted by transfecting JB6 cells with ARE-luciferase reporter plasmid (Fig. 4C). 17-Oxo-DHA induced a time-dependent increase in luciferase activity, which peaked at 6 h. 17-oxo-DHA treatment caused a twofold increase in transcriptional activity of Nrf2, whereas DHA produced only a slight increase in the activity, lending further support to our view that 17-oxo-DHA is more potent ARE inducer than DHA.

17-Oxo-DHA-induced upregulation of HO-1 requires presence of Nrf2

To further verify whether 17-oxo-DHA-induced upregulation of HO-1 is dependent on the activation of Nrf2, Nrf2 was silenced by transfecting JB6 cells with Nrf2 siRNA, and the expression of HO-1 following 17-oxo-DHA treatment was measured by Western blot analysis (Fig. 5A). As compared to cells transfected with scrambled siRNA, 17-oxo-DHA-induced expression of HO-1 was abrogated in cells transfected with siNrf2, indicating that Nrf2 mediates 17-oxo-DHA-induced upregulation of HO-1 expression in mouse epidermal cells. Furthermore, embryonic fibroblasts obtained from Nrf2 wild-type and knockout mice (MEFs) were treated with 17-oxo-DHA and the expression of HO-1 protein was compared to untreated control by Western blot analysis (Fig. 5B). 17-Oxo-DHA induced the expression of HO-1 in wild-type MEF cells whereas no induction was observed in Nrf2 knockout MEF cells. Thus, it is evident that Nrf2 mediates 17-oxo-DHA-induced upregulation of HO-1 expression in mouse epidermal cells.

17-Oxo-DHA decreases the steady state level of Keap1

The activity of Nrf2 is mainly regulated by Keap1, which is a substrate adaptor protein for Cul3-dependent ubiquitin ligase complex that represses Nrf2 by targeting Nrf2 for ubiquitination and subsequent proteasomal degradation[5, 13, 35]. Recent studies have shown that certain electrophiles can cause switching of Cul3-dependent ubiquitination of Nrf2 to Keap1, thereby targeting Keap1, not Nrf2, for ubiquitination and subsequent degradation[9, 36]. To determine whether the Keap1 protein level in JB6 cells is affected by 17-oxo-DHA, we examined the *Keap1* mRNA and Keap1 protein levels in 17-oxo-DHA treated JB6 cells. 17-Oxo-DHA markedly decreased steady-state levels of Keap1 protein in a time-dependent manner (Fig. 6A), whereas its mRNA level remained constant (Fig. 6B). Consistent with the results on Nrf2, the degradation of Keap1 was more pronounced in cells treated with 17-oxo-DHA than that achieved with DHA (Fig.6C). Corroboratory results were obtained with HEK 293T cells transfected with expression vectors for wild-type Flag-Keap1 whereby treatment of 17-oxo-DHA markedly reduced steady-state level of Keap1 (Fig. 6D). To determine if Keap1 is ubiquitinated in response to 17-oxo-DHA, JB6 cells were treated with 17-oxo-DHA for 6 h or 12 h, lysed and subjected to immunoprecipitation with Keap1 antibody. The presence of ubiquitin-conjugated Keap1 proteins was assessed by

immunoblot analysis of anti-Keap1 immunoprecipitates with anti-ubiquitin antibody. As shown in Fig. 6E, 17-oxo-DHA markedly increases Keap1 ubiquitination in JB6 cells.

Effects of Keap1 C151S, C273S, and C288S mutation on 17-oxo-DHA-induced activation of Nrf2-dependent transcription

Keap1 is a cysteine rich protein, with 27 cysteine residues in the human and 25 cysteine residues in murine protein. Figure 1B represents a structure of 17-oxo-DHA (*left*), which harbors the α,β unsaturated carbonyl moiety that can serve as an electrophile toward cellular nucleophiles. Recent studies have reported that electrophiles can modify reactive cysteines in Keap1, which leads to disruption of the interaction between Keap1-Nrf2, thereby promoting Nrf2 nuclear accumulation and ARE activation[34]. To determine if 17-oxo-DHA induces Nrf2 activation through direct Keap1 cysteine thiol modification, dithiothreitol (DTT) was utilized as a disulfide reducing agent. JB6 cells were treated with 17-oxo-DHA in the presence or absence of DTT, and the cells were lysed and subjected to Western blot analysis. As shown from the result, treatment of DTT significantly attenuated 17-oxo-DHA–induced expression of HO-1 (Fig. 7A), indicating that direct modification of Keap1 cysteine thiol is a principle mode of action whereby 17-oxo-DHA activates Nrf2.

Several studies have identified 3 critical cysteine residues present in Keap1–cys151, 273, and 288—that are speculated to be involved in regulating the activity of Nrf2[34]. In order to determine which cysteine residue in Keap1is involved in the activation of Nrf2 following 17-oxo-DHA treatment, we utilized expression vectors for Keap1 mutants-C151S, C273S, C288S- in which each cysteine residues were converted to serine. HEK 293T cells transfected with wild-type or mutant Keap1 expression vectors were treated with 17-oxo-DHA, and the expression levels of Nrf2 and HO-1 protein were analyzed by Western blot analysis. Our results show that 17-oxo-DHA–induced activation of Nrf2 and concurrent expression of HO-1 are abated in cells harboring Keap1 C151S mutation (Fig. 7B), suggesting that Cys151 residue of Keap1 serves as a critical role in mediating 17-oxo-DHA induced expression of HO-1. In addition, attenuated expression of HO-1 was also observed in cells harboring Keap1 C273S mutation. However, their Nrf2 expression appeared to be constantly upregulated. Interestingly, substantial reduction in Keap1 expression level upon

17-oxo-DHA treatment was observed in cells harboring wild-type Keap1, whereas the Keap1 level remained unchanged in cells harboring mutant Keap1.

Furthermore, based on our previous observation that 17-oxo-DHA induces ubiquitination of Keap1, and that Cys151—and possibly Cys273—are the potential target residues for 17-oxo-DHA, I attempted to find out whether these cysteine residues are also involved in the 17-oxo-DHA-induced ubiquitination of Keap1. In order to determine the role of cysteine residues in 17-oxo-DHA-induced ubiquitination of Keap1, HEK293T cells transfected with expression vectors for wild-type, Keap1-C151S, or Keap1-C273S were treated with or without 17-oxo-DHA, and the lysates were subjected to immunoprecipitation with Keap1 antibodies. The presence of ubiquitinated Keap1 proteins was analyzed by immunoblot analysis of anti-Keap1 immunoprecipitates with anti-ubiquitin antibody. Increase in ubiquitination of Keap1 was only observed in the cells harboring wild-type Keap1 (Fig. 7C), suggesting that Cys151 and Cys273 are also required for 17-oxo-DHA-induced ubiquitination of Keap1.

In the course of our analysis, I also found that 17-oxo-DHA treatment resulted in formation of a high molecular weight (HMW) form of Keap1 with a molecular mass of about 170 kDa. These HMW bands were detected in 17-oxo-DHA-treated cells harboring wild-type Keap1 or Keap1-C273S (Fig. 7D; *lane 2 and 6*), whereas expression of this HMW form of Keap1 was completely abolished in cells harboring Keap1-C151S (Fig. 7D; *lane 4*). Taken together, these results suggest that Cys151 may also play an important role in formation of this HMW form of Keap1 in response to 17-oxo-DHA.

Docking model of 17-oxo-DHA and Keap1 show predicted interaction at Cys151

Several electrophiles have been reported to covalently modify cysteine residues of Keap1 and disrupt the interaction between BTB domain of Keap1 and Cul3[7, 25]. A recent study has identified the structure of Keap1 BTB domain in complex with CDDO, which is a well-studied antagonist of Keap1[3]. Here, Cleasby et al., provides structural confirmation of covalent interaction between CDDO and Cys151 of Keap1 BTB domain, suggesting that adduction of Cys151 by CDDO disrupts the BTB/Cul3 interaction. We conducted a docking study to examine whether 17-oxo-DHA has the potential to bind Cys151, and AutoDock Vina program was used to determine a docking mode of 17-oxo-DHA into BTB

domain of Keap1. To obtain a model for the bound complex of Keap1 and 17-oxo-DHA, we used the structure of Keap1 BTB domain in the CDDO-bound conformation, and 17-oxo-DHA structure was docked on the binding site in place of CDDO (Fig 8.A). The predicted binding affinity value in this model was -3.0 kcal/mol, with 3.9 Å distance between beta carbon of the 17-oxo-DHA and Cys151 residue of Keap1 BTB domain. To examine if the change in Keap1 structural conformation followed by 17-oxo-DHA-induced covalent modification of Cys151 affects the interaction between Cul3 and Keap1, the computational simulation of a binding between compound-bound Keap1 structure and Cul3 was conducted (Fig. 8B and C). Since the crystallographic information for Keap1 structure is limited, we used the structure of its related protein KLHL11 to predict the structure of Keap1-Cul3 complex. The structure of a CDDO-bound Keap1 complex was superimposed on the previously solved structures of the KLHL11-Cul3 complex[1]. Compared with the unbound structure of KLHL11, our results indicated that the covalent modification of Cys151 by CDDO—and possibly by 17-oxo-DHA—can induce conformational shift in the alpha-helix of the BTB domain, and thereby affect the binding interface between Keap1 BTB domain and Cul3.

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

A question that still needs to be addressed is how Keap1 protein is degraded. It has been reported that ubiquitinated Keap1 proteins in response to certain electrophiles undergo degradation via a proteasome-independent pathway[36]. In agreement with this report, recent study has also demonstrated that Keap1 degradation is associated with p62-mediated autophagy[30]. p62 plays an important role in autophagy as a substrate carrier, and it is known for its ability to bind numerous proteins including polyubiquitinated proteins[30]. Based on our previous result that 17-oxo-DHA induces ubiquitination of Keap1, the possible involvement of p62 in degradation of ubiquitin-conjugated Keap1 was examined following 17-oxo-DHA treatment. The levels of *p62* mRNA and p62 protein in the 17-oxo-DHA treated JB6 cells were measured by RT-PCR and Western blot analysis. A time-dependent increase in *p62* mRNA (Fig. 9A) and protein level (Fig. 9B) was observed upon treatment with 17-oxo-DHA, which coincided with the time-dependent decreasing tendency of Keap1 protein level following 17-oxo-DHA treatment. To further determine if 17-oxo-DHA-

induced ubiquitination and degradation of Keap1 occur via p62-mediated autophagy, I examined the Keap1 protein level in JB6 cells in response to 17-oxo-DHA in the presence of autophagy inhibitor, bafilomycin. In the absence of the bafilomycin, 17-oxo-DHA treatment caused marked reduction in Keap1 protein level (Fig. 9C). When 17-oxo-DHA was treated in the presence of bafilomycin, the level of Keap1 was restored. The elevated level of LC3 protein in bafilomycin treated cells indicate a successful inhibition of autophagy. Taken together, these results further support the notion that Keap1 is degraded via p62-mediated autophagy.

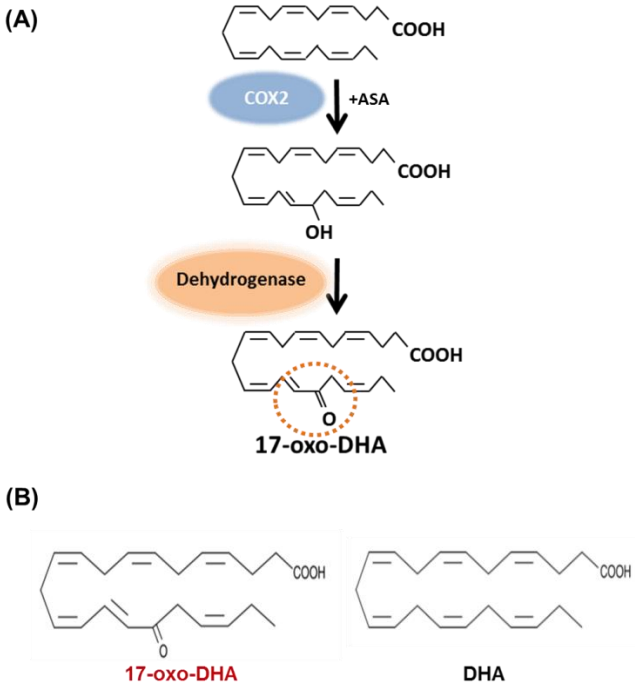

Figure 1. 17-Oxo-DHA is an electrophilic metabolite of DHA. (A) 17-Oxo-DHA, an electrophilic derivative of DHA, is generated by COX-2 catalyzed mechanism in activated macrophages. Modulation of COX-2 activity by aspirin (ASA) increases the rate of electrophilic metabolite production. (B) 17-Oxo-DHA (*left*) harbors an α,β unsaturated carbonyl moeity which confers electrophilic activity towards cellular nucleophiles, whereas DHA (*right*) is a non-electrophilic molecule.

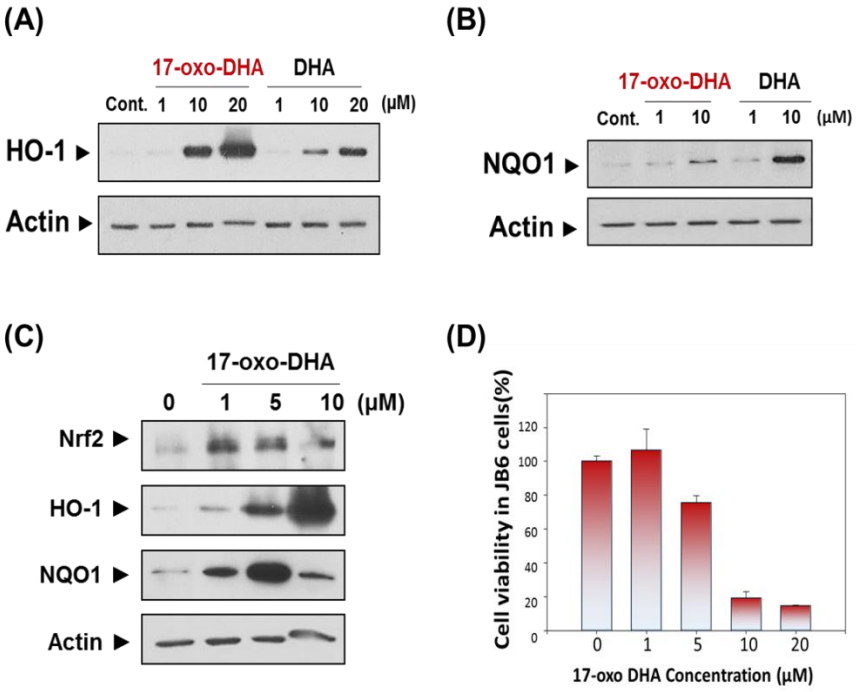

Figure 2. 17-Oxo-DHA is a more potent inducer of HO-1 expression than DHA. (A, B) JB6 cells were treated with 1-20 μM of 17-oxo-DHA or DHA for 24 hours. Total proteins were isolated and the expression levels of HO-1 and NQO1 were analyzed by Western blot analysis. (C) JB6 cells were treated with 1-10 μM of 17-oxo-DHA for 24 hours, and total protein were isolated and expression levels of Nrf2 and its target proteins were analyzed by Western blot analysis. (D) JB6 cells were seeded in 48-well plates at a density of 3 x 10^3 in 300μl media per well, and the next day cells were treated with indicated concentrations of 17-oxo-DHA for 24 hours. After incubation for 24 h, cells were incubated with MTT solution (0.5 mg/ml) for 2 h and the absorbance at 570 nm was read using a microplate reader.

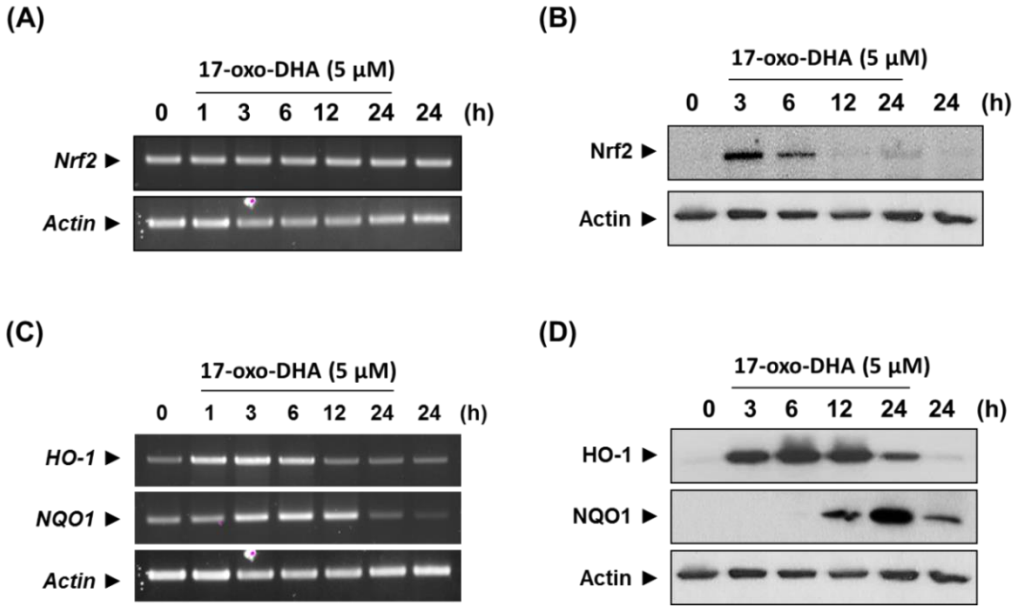

Figure 3. 17-Oxo-DHA induces expression of Nrf2 and their target proteins in JB6 cells. (A and B) JB6 cells were treated with 5 μM of 17-oxo-DHA for indicated time periods, and total RNA and protein were isolated. The mRNA level and proteins expression level of Nrf2 were analyzed by RT-PCR (A) and Western blot analysis (B), respectively. (C and D) JB6 cells were treated with 5 μM of 17-oxo-DHA for indicated time periods, and total RNA and protein were isolated. The mRNA level and proteins expression level of Nrf2 target proteins, HO-1 and NQO1, were analyzed by RT-PCR (C) and Western blot analysis (D), respectively

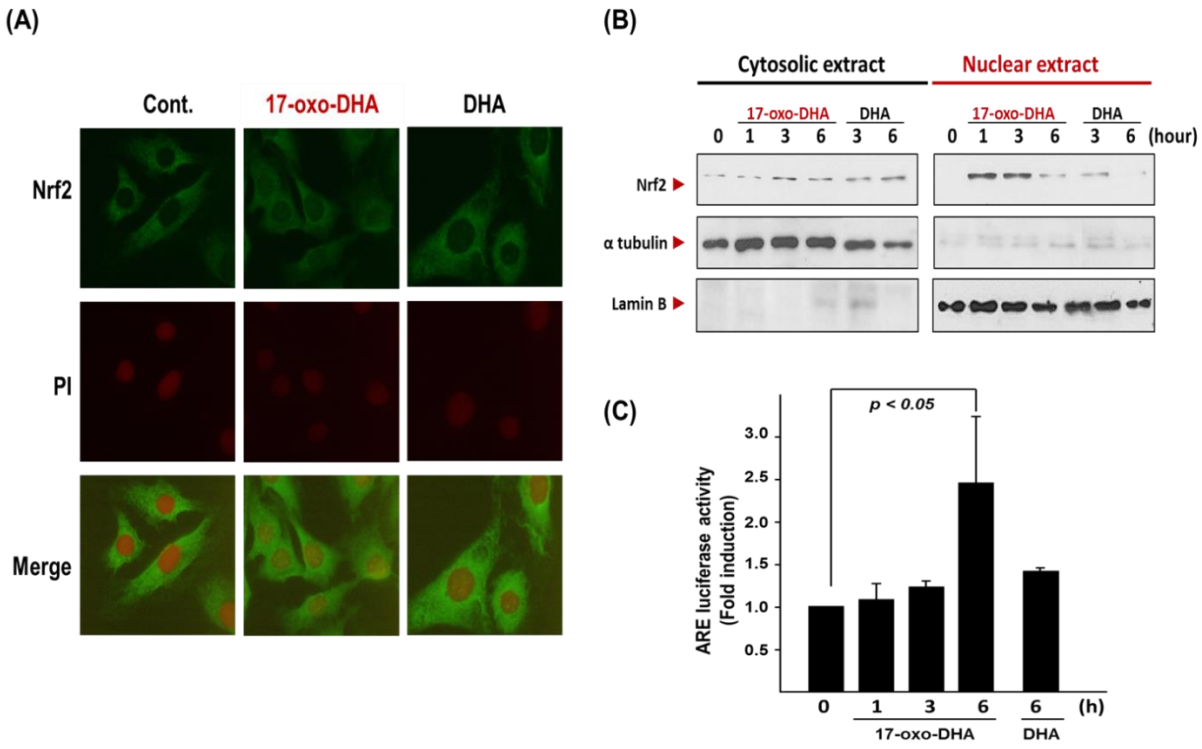

Figure 4. 17-Oxo-DHA is a more potent ARE inducer than DHA. (A) JB6 cells were seeded into 4-well chamber slides at a density of 1 X 10^4 per well, and treated with 5 μM 17-oxo-DHA or 5 μM DHA for 3 hours. After 3 h, cells were subjected to fixation and permeabilization. The cells were incubated with 5 % BSA for 1 h and incubated with Nrf2 primary antibody overnight. Next day, cells were incubated with FITC-conjugated secondary antibody for 1 h. Cells were also counterstained with propodium iodide (PI) and examined under a fluorescent microscope. (B) JB6 cells were treated with 5 μM 17-oxo-DHA or 5 μM DHA and harvested at the indicated times. Nuclear fraction and cytosolic fraction were isolated from cells and the nuclear Nrf2 levels were analyzed by Western blot analysis. (C) JB6 cells were seeded into 6-well plate at a density of 8 x 10^4 per well. Next day, 2 μg of ARE-luciferase plasmid and 0.5 μg of β-galactosidase vector was transfected for 24 h. After 24 h, cells were treated with 5 μM of 17-oxo-DHA or 5 μM of DHA and harvested after indicated time periods. The cells were then lysed and analyzed for luciferase activity using a microplate luminometer. The β-galactosidase activity was also determined by spectrophotometry (OD 420).

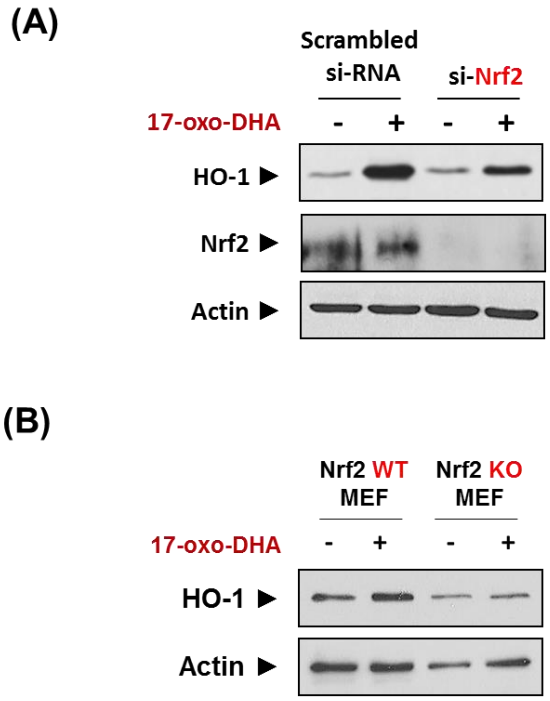

Figure 5. 17-Oxo-DHA-induced upregulation of HO-1 requires presence of Nrf2.

(A) JB6 cells were transfected with 20 nM of the Nrf2 siRNAs for 36 h, and the cells were subjected to starvation with media containing 0.5 % serum for 12 h. After starvation, cells were treated with 5 μM of 17-oxo-DHA and harvested after 6 h. Total protein was isolated and the expression level of Nrf2 protein and its target protein HO-1 was analyzed by Western blot analysis. (B) Nrf2-wild type or Nrf2-Knockout MEF cells were incubated with or without 5 μM 17-oxo-DHA and harvested after 6 hours. Total protein was isolated and HO-1 protein expression level was analyzed by Western blot analysis.

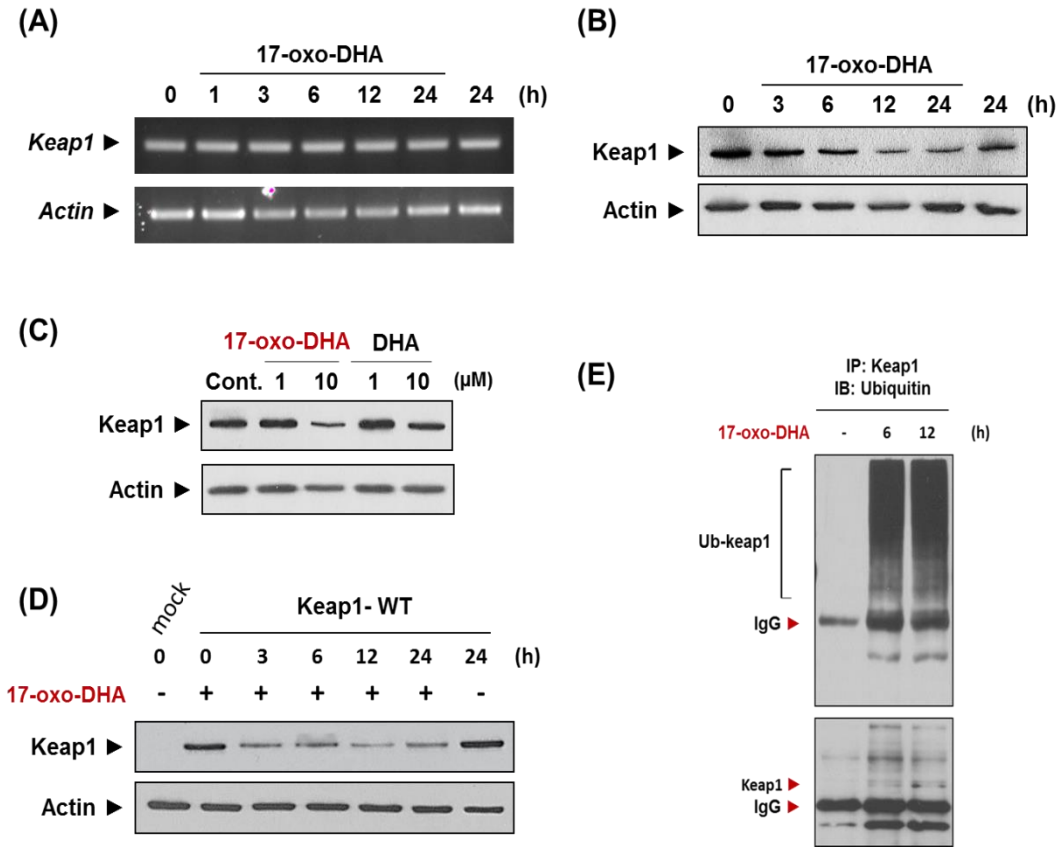

Figure 6. 17-Oxo-DHA decreases the steady state level of Keap1. (A and B) JB6 cells were treated with 5 μM of 17-oxo-DHA for indicated time periods, and Keap1 mRNA level and protein expression level were analyzed by RT-PCR (A) and Western blot analysis (B), respectively. (C) JB6 cells were treated with 1 or 10 μM of 17-oxo-DHA and 1 or 10 μM of DHA for 24 hours. Total proteins were isolated and the expression levels of Keap1 were analyzed by Western blot analysis. (D) HEK293T cells were transfected with wild-type Flag-Keap1 expression vector for 36 h. After 36 h, cells were treated with 5 μM of 17-oxo-DHA for indicated time periods. Total protein was isolated and Keap1 protein expression level was analyzed by Western blot analysis. (E) JB6 cells were treated with 5 μM of 17-oxo-DHA and harvested after indicated time intervals. The cell lysate was subjected to immunoprecipitation with Keap1 antibodies and anti-Keap1 immunoprecipitates were subjected to immunoblot analysis with anti-ubiquitin antibodies (*upper panel*) or with anti-Keap1 antibodies (*bottom panel*)

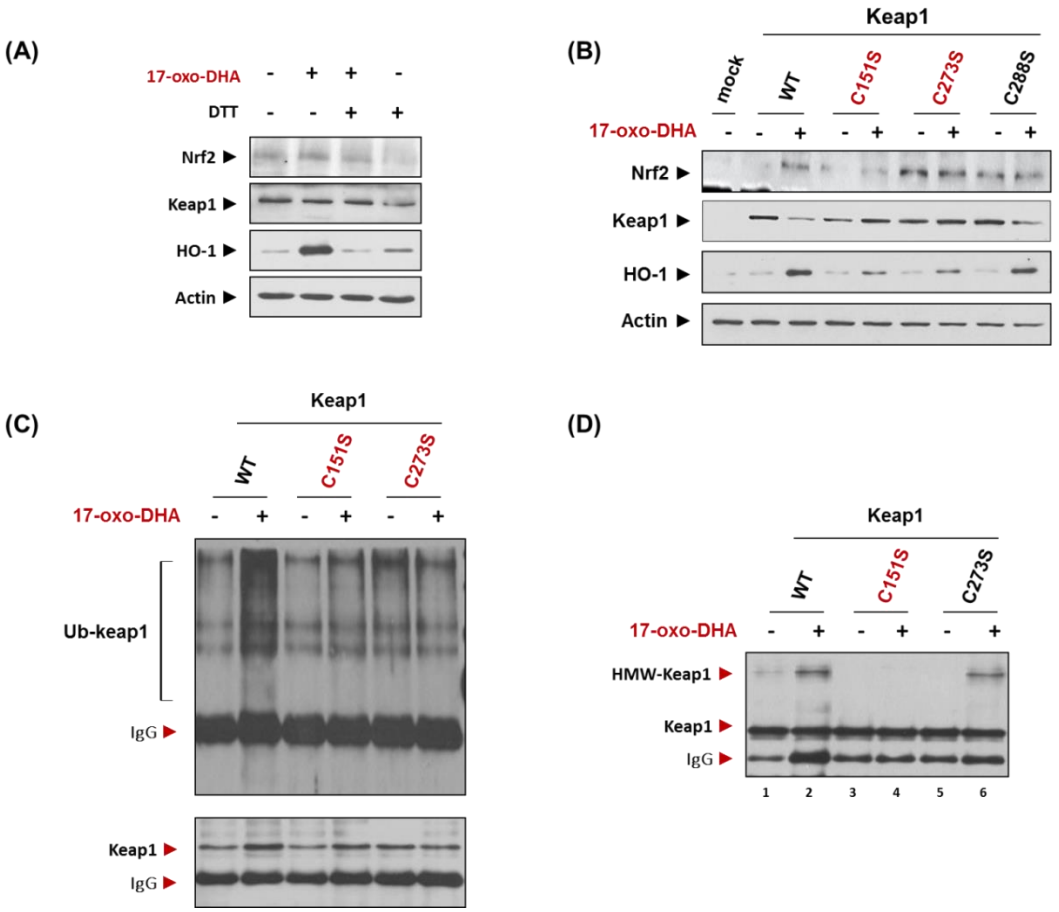

Figure 7. Effects of Keap1 C151S, C273S, and C288S mutation on 17-oxo-DHA-induced activation of Nrf2-dependent transcription and ubiquitination of Keap1 (A) JB6 cells were pre-incubated with or without DTT (100 μM) for 1 h and treated with 5 μM of 17-oxo-DHA for 6 h. Total proteins were isolated and expression level of Nrf2, Keap1 and HO-1 protein were analyzed by Western blot analysis. (B) HEK293T cells were transfected with 5μg of expression vectors for wild-type Flag-Keap1 or mutant Keap1 proteins for 36 h. After 36 h, cells were treated with or without 17-oxo-DHA and were harvested after 12 hours. Total protein was isolated and HO-1 expression level was analyzed by Western blot analysis. (C and D) HEK293T cells were transfected with expression vectors for either wild-type Flag-Keap1 or mutant Keap1 proteins for 36 h. Cells were treated with or without 17-oxo-DHA for 12 h, lysed, and subjected to immunoprecipitation with anti-Keap1 antibodies. Anti-Keap1 immunoprecipitates were subjected to immunoblot analysis with anti-Ubiquitin antibodies (C; *upper panel*) or with anti-Keap1 antibodies (C;*bottom panel, D*).

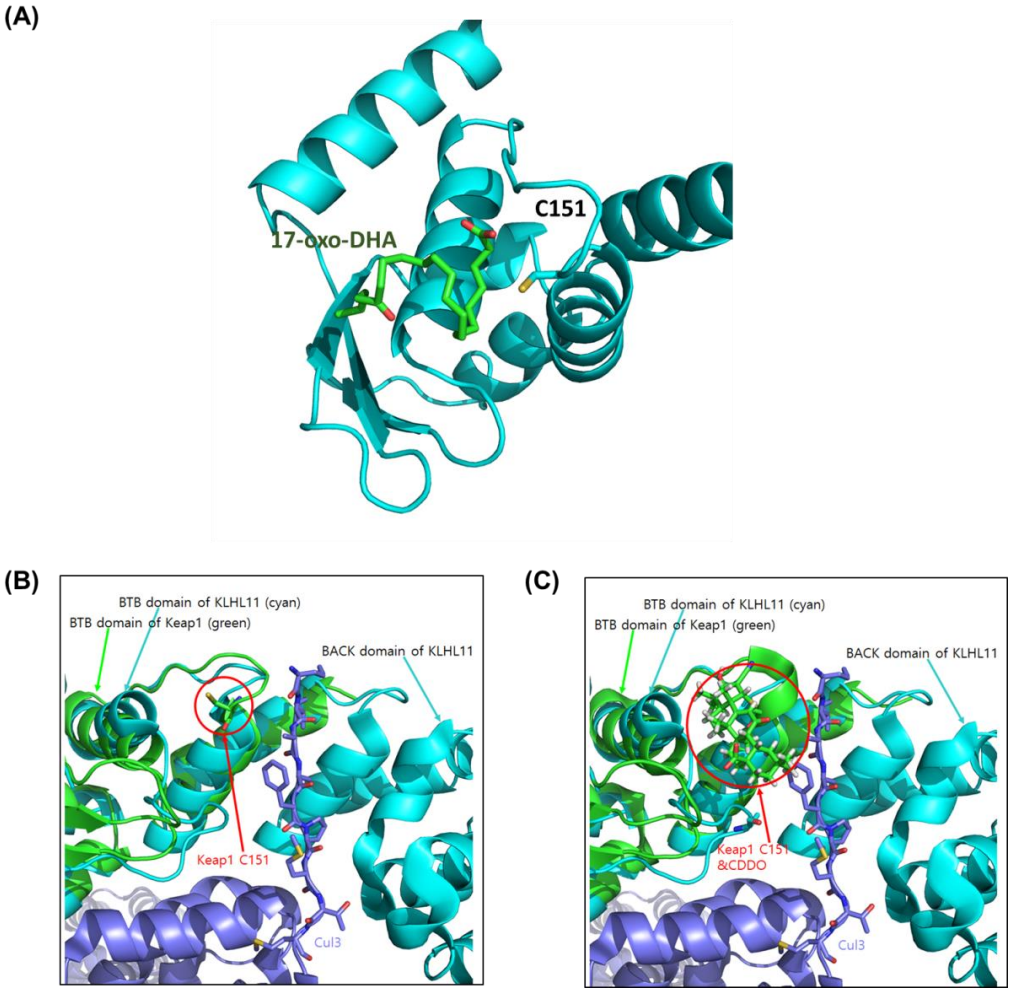

Figure 8. Cys151 is a putative target of 17-oxo-DHA. (A) Docking model of 17-oxo-DHA and Keap1 show predicted interaction at Cys151 of Keap1 BTB domain. (B and C) Computational simulation of a binding between Cul3 and Keap1 structure alone (B) or CDDO-bound structure of Keap1(C).

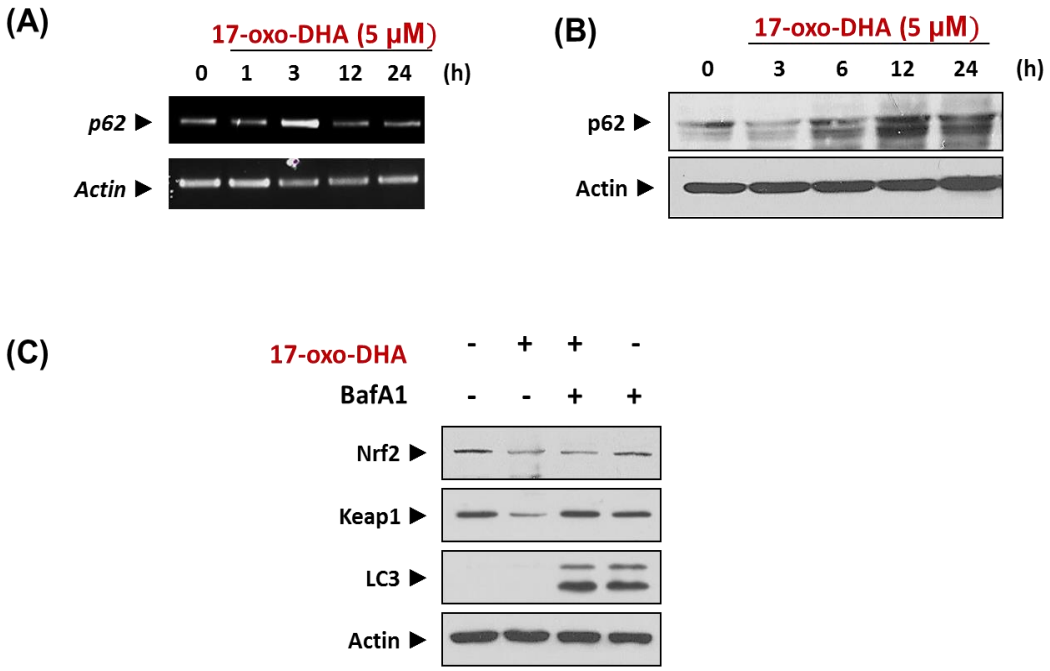

Figure 9. 17-Oxo-DHA induces degradation of Keap1 through p62-mediated autophagy. (A and B) JB6 cells were treated with 5 μM of 17-oxo-DHA for indicated time periods, and p62 mRNA level and protein expression level were analyzed by RT-PCR (A) and Western blot analysis (B), respectively. (C) JB6 cells were incubated with 1 μM of Bafilomycin A1(BafA1) for 1 h prior to treatment of 17-oxo-DHA. Cells were treated with or without 5 μM of 17-Oxo-DHA in the presence or absence of BafA1 for 12 h. The expression levels of Nrf2, Keap1, and LC3 protein were determined by Western blot analysis.

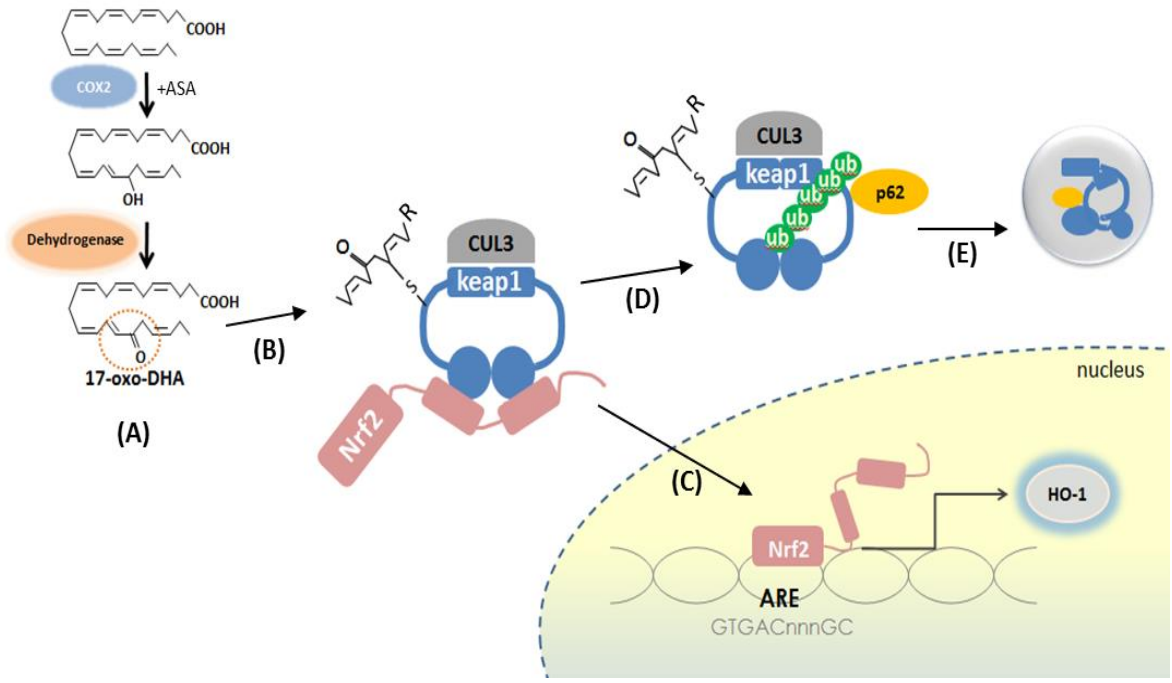

Figure 10. Schematic representation of the action of 17-oxo-DHA in JB6 cells. (A-E) 17-oxo-DHA is an electrophilic oxo-derivative of DHA (A). It harbors α,β unsaturated carbonyl moiety that is reactive towards cellular nucleophiles such as cysteine residues of Keap1 (B). Modification of specific cysteine residues of Keap1 leads to stabilization of Nrf2, which translocates into the nucleus and promotes transcription of its target protein, HO-1 (C). 17-Oxo-DHA-induced modification of Keap1 cysteine residues also lead to Keap1 ubiquitination (D), targeting Keap1 for degradation through p62-mediated autophagy (E).

DISCUSSION

Oxidative stress is a key pathogenic factor implicated in progression of various diseases including cancer. Significant efforts have been devoted to identify biological electrophiles and to elucidate how these particular species can activate the cellular antioxidant response to combat the deleterious effects of ROS-mediated oxidative damage.

Groeger *et al.* identified new class of electrophilic oxo-derivatives (EFOX) that are generated by the action of COX-2 and dehydrogenase in activated macrophages, underscoring its potential as bioactive lipid mediators that can modulate several cellular signaling pathways including Nrf2-dependent anti-oxidant response[8]. The major function of Nrf2 is to activate cellular defense mechanism by triggering the expression of a battery of cytoprotective genes to protect the cells against carcinogenic insults. The present study was conducted in an attempt to fully elucidate the molecular mechanism underlying the antioxidant response induced by electrophilic fatty acids in normal cells in the context of their potential as chemopreventive agents. In this study, we focused on 17-oxo-DHA, a mono-oxygenated electrophilic derivative of DHA, and sought to investigate its biological effect in mouse epidermal JB6 cells. Our results clearly demonstrate that 17-oxo-DHA significantly enhances the expression of antioxidant enzymes HO-1 and NQO1 in mouse epidermal JB6 cells. Previous studies have demonstrated the antioxidative and anti-inflammatory properties of DHA[2, 16, 18, 33], and we questioned whether 17-oxo-DHA and its precursor DHA produce comparable effects in JB6 cells. Here, we provide an important finding that although both lipids elicit similar effects, 17-oxo-DHA is more potent ARE inducer than DHA. The electrophilic nature of 17-oxo-DHA, as opposed to non-electrophilic DHA, may account for its ability to trigger more substantial effect. DHA-induced HO-1 expression is also known to be mediated by Nrf2, but the upstream signaling pathways that leads to Nrf2 activation involve early stage generation of ROS and activation of PI3K/Akt, ERK1/2 and p38 signaling pathways[33]. On the other hand, like many of the widely known electrophilic Nrf2 activators, we have shown that 17-oxo-DHA modulates Nrf2 signaling by directly modifying reactive cysteine residues present in Keap1. Our speculation that Keap1 thiol modification by 17-oxo-DHA induces Nrf2-dependent expression of HO-1 was supported by our experimental evidence. The ability of DTT to

block 17-oxo-DHA-induced expression of HO-1 indicates that DTT hinders the interaction between 17-oxo-DHA and critical cysteine residues of Keap1 required for regulation of Nrf2 activity.

Recently, extensive studies have been done to elucidate the mechanism whereby cysteine modifications in Keap1 lead to Nrf2 activation. Although the exact mechanism is not known, the proposed mechanisms share one important perspective: Keap1 is not just a passive repressor that sequesters Nrf2 in the cytoplasm, but rather an active mediator that targets Nrf2 for ubiquitination and proteasomal degradation[5, 13, 35, 37]. It is now widely recognized that Keap1 functions as a substrate adaptor protein for Cul3-dependent E3 ubiquitin ligase complex[5, 35]. Based on the knowledge that Keap1 is rich in cysteine residues that are reactive toward electrophiles, and that electrophiles activate Nrf2 by inhibiting Keap1-directed ubiquitination of Nrf2, we postulated that 17-oxo-DHA may also interact with particular cysteine residues and induce conformational change of Keap1, resulting in Nrf2 stabilization by inhibiting ubiquitination of Nrf2. Several reactive cysteine residues have been under investigation and the mutation studies have identified 3 critical cysteine residues that are important for regulation of Nrf2 activity: Cys151, Cys273, and Cys288[34]. In particular, mutation of Cys151 to serine has been shown to abolish electrophile- and oxidant-mediated Nrf2 ubiquitination[34]. Consistent with this report, our results indicate that Cys151 is required for stabilization of Nrf2 in response to 17-oxo-DHA. Two cysteine residues, Cys273 and Cys288 are reported to be required for Keap1-dependent ubiquitination of Nrf2, and their ability to repress Nrf2-dependent transcriptional activation was impaired in mutant Keap1-C273S and C288S[34]. The uncertain aspect of our result that requires further investigation is that while mutation of Cys151 abolishes the activation of Nrf2 and concurrent expression of its target protein HO-1, mutation of Cys273 results in attenuated expression HO-1 in spite of the constitutively active Nrf2. It is likely that although C273S mutation of Keap1 impairs its ability to repress Nrf2, other post-translational modifications of Nrf2 in response to 17-oxo-DHA treatment may affect the nuclear accumulation of Nrf2, and may account for the attenuated expression of HO-1.

Based on our results, we speculate that either Cys151 or Cys273 may be a molecular target of 17-oxo-DHA. Relevant to this aspect, another important finding in our results is that Keap1 is ubiquitinated and degraded in response to 17-oxo-DHA, and both Cys151 and

Cys273 are required for 17-oxo-DHA-induced ubiquitination and subsequent degradation of Keap1. Interestingly, we also found that Cys151 in Keap1 are required for formation of high molecular weight (HMW) form of Keap1 in cells treated with 17-oxo-DHA. Cysteine 151 resides in the N-terminal BTB domain of Keap1, the domain that is speculated to interact with Cul3[5, 6]. Several works have shown that certain electrophiles and oxidants can induce dissociation of Keap1 from Cul3 complex, thereby inhibiting Nrf2 ubiquitination and subsequent degradation[7, 35]. Based on these previous studies and our results, we propose two possible mechanisms for 17-oxo-DHA-induced activation of Nrf2 dependent transcription in JB6 cells. 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 in JB6 cells. Moreover, Zhang *et al*., have 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[34]. On the basis of these findings, the second possible mechanism we propose is that 17-oxo-DHA modulates the Cys273 of Keap1 and thereby inhibit Keap1-mediated ubiquitination of Nrf2, resulting in Nrf2 stabilization and upregulation of Nrf2-dependent expression of HO-1.

Interestingly, concerning 17-oxo-DHA-induced Keap1 ubiquitination, a relevant study has found that mutations within BTB domain of Keap1 at the predicted interface between BTB and Cul3 decreases ubiquitination of Nrf2 but increase Keap1 ubiquitination[35]. Since Cys151 is located in the BTB domain, we surmised that modification of Cys151 by 17-oxo-DHA may lead to a conformational change of Keap1 and concomitant alteration in the interaction between the Keap1 and Cul3 complex, thereby inducing a substrate switch from the Nrf2 to Keap1. This idea was in support of the finding that Keap1 can be ubiquitinated by the same Cul3-Rbx1 complex that ubiquitinates Nrf2[36]. Taken together, these results support our proposed mechanism in which Cys151 modification by 17-oxo-DHA reduces the association between Keap1 and Cul3, thereby inhibiting Keap1-dependent ubiquitination of Nrf2. Thus, although speculative at this point, we conclude that 17-oxo-DHA-induced modification of Cys151 or Cys273 leads to activation of Nrf2 by inhibiting Keap1-dependent ubiquitination of Nrf2. The conformational shift of Keap1 following Cys151 modification by 17-oxo-DHA may lead to

substrate switch such that Keap1, instead of Nrf2, becomes the target of Cul3-mediated ubiquitination.

In line with the work of Zhang et al.,[34] our results indicate that 17-oxo-DHA induces Cys151-dependent post-translational modification of Keap1. Although the nature of the HMW form of Keap1 is not clear, it has been suggested that it could be covalently adducted protein from two different proteins or a dimeric form of Keap1[37]. An interesting finding in our study with respect to this view is that 17-oxo-DHA induces upregulation of p62, which is a scaffold protein that targets protein aggregates for degradation via autophagy[11]. Several studies have suggested that p62 can modulate the activity of Nrf2 by directly interacting with Keap1, thereby disrupting the formation of Keap1-Nrf2 complex[4, 11, 15]. In support of a previous study which demonstrates that certain electrophiles accelerate the degradation of Keap1 by autophagy[30], our results also indicate that Keap1undergoes degradation via an autophagy pathway in response to 17-oxo-DHA treatment. Based on our aforementioned results in conjunction with previous studies, it is conceivable that Cys151-dependent conformational change of Keap1 by 17-oxo-DHA may render this modified Keap1 more susceptible to ubiquitin conjugation, making it a preferable target for p62-mediated autophagy. Since p62 not only harbors ubiquitin-associated domain (UBA) but also KIR motif through which p62 directly interacts with Keap1, we speculate that the binding of the Keap1 and p62 may account for formation of HMW form of Keap1. However, identifying the nature of a HMW form requires further investigation.

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국문초록

세포 내 산화적 스트레스는 활성산소종의 생성과 생체 내 방어기전 간의 불균형으로 인한 결과이다. 이러한 산화적 스트레스가 제어 되지 않을 경우 암을 비롯한 여러 인체 질병을 일으키는 주요한 원인이 되는 것으로 알려져 있다. 따라서 세포 내외에서 비롯된 다양한 유독한 물질을 제거 하고 이에 대항하는 방어기전을 효과적으로 활성화 시킬 수 있는 물질을 발견하는 것이 중요한 과제로 대두 되고 있다.

생체 내 방어기전을 조절하는데 있어 대표적인 역할을 하는 분자인 Nrf2 는 세포보호 단백질이나 항산화 효소들의 발현을 촉진 시키는 전사 인자로 알려져 있다. 따라서 이러한 기능을 갖는 Nrf2 의 활성을 지속적으로 증진시킬 수 있는 천연 또는 합성 물질에 대한 연구가 중요하다.

DHA 와 같은 오메가-3 지방산의 산화적 스트레스에 대한 보호 효과가 선행연구에서 입증된 바 있다. 본 연구에서 이용된 17-oxo-DHA 는 최근 새롭게 밝혀진 DHA 의 대사산물로서, COX-2 와 dehydrogenase 에 의해 생성되며 α,β 불포화 카보닐기 구조를 포함한 친전자체 물질이다. 마우스 상피세포인 JB6 세포에 17-oxo-DHA 를 처리 하였을 때, 주요 항산화 효소인 HO-1 단백질의 발현이 시간과 농도 의존적으로 확연히 증가함을 확인 하였다. 또한 Luciferase assay 를 통해 17-oxo-DHA 가 ARE 를 포함하고 있는 마우스 ho-1 프로모터를 활성화 시킨다는 것을 하였으며, immunocytochemistry 와 세포 분획을 통하여 17-oxo-DHA 가 Nrf2 의 핵 내 이동을 증가시키는 것을 관찰 하였다. 또한 위 실험 결과들을 동일한 조건 하에 DHA 를 처리한 JB6 세포에서 얻은 실험결과들과 비교하였을 때, 17-oxo-DHA 가 기존에 알려진 DHA 의 효과 보다 더욱 강력한 분자적 방어 기전의 매개체임이 확인되었다.

더 나아가, JB6 세포에 Nrf2 siRNA 를 도입 하였을 때 17-oxo-DHA 에 의해 유도된 HO-1 의 발현이 감소함을 관찰함으로써 Nrf2 가 17-oxo-DHA 에 의한 HO-1 단백질의 발현을 매개한다는 것을 확인 하였다. 이와 마찬가지로 Nrf2 유전자가 결핍된 MEF(Mouse Embryonic Fibroblast) 세포에서도 17-oxo-DHA 를 처리 하여 HO-1 발현을 비교하였을 때 Nrf2 가 정상적으로 존재하는 세포에 비해 발현 정도가 감소하는 것을 관찰 하였다.

Keap1 은 Cul3-Rbx1 ubiquitin ligase 와 함께 Nrf2 의 활성을 조절하는 단백질로서, 보통 상태에서는 Nr2 의 핵 내 이동을 막고 Nrf2 를 지속적으로 ubiquitin 과

결합시켜 proteasome 에 의한 분해를 유도한다. 그러나 친전자체나 산화적 자극에 의한 Keap1 의 주요 Cysteine 잔기의 변형이 Keap1 의 구조적 변형으로 이어지며 Cul3 과의 상호작용의 변화를 일으켜 Nrf2 는 더 이상 ubiquitination 되지 않고 핵 내로 이동할 수 있게 된다. 환원제인 DTT 를 함께 처리하였을 때 17-oxo-DHA 에 의한 HO-1 의 발현이 감소됨을 관찰함 으로써 친전자성 17-oxo-DHA 와 친핵성인 Cysteine 잔기와의 결합 가능성을 확인 하였다. 또한 Keap1 의 Cysteine 잔기들 중 Nrf2 의 활성을 조절하는데 중요하다고 알려진 C151, C273, 그리고 C288 잔기가 17-oxo-DHA 에 의한 Nrf2 의 안정화 과정에도 중요한 역할을 하는지 알아보기 위해 세포에 wild-type Keap1 또는 mutant Keap1 을 과발현 시켜 17-oxo-DHA 에 의한 HO-1 의 발현을 측정하였다. 그 결과 C151 잔기를 변형시킨 Keap1 이 과발현된 세포와 C273 잔기가 변형된 Keap1 이 과발현된 세포에서 각각 HO-1 의 발현 정도가 현저히 감소되었고, 이로부터 Keap1 의 C151 번과 C273 번 잔기와 17-oxo-DHA 과의 결합 가능성을 추측할 수 있었다.

17-oxo-DHA 를 이용한 본 연구에서의 또 하나 중요한 관점은 17-oxo-DHA 가 앞서 언급한 cysteine 잔기들과의 상호작용을 통하여 Keap1 의 ubiquitination 와 분해 또한 유도한다는 것이다. 그러나 proteasome 에 의해 분해되는 Nrf2 와는 달리 Keap1 은 autophagy 에 의해 분해된다는 보고를 바탕으로 실험한 결과, 17-oxo-DHA 에 의해 autophagy 를 매개하는 주요한 인자인 p62 단백질의 발현이 유도됨을 확인하였다. 또한 autophagy 의 저해제인 bafilomycin 을 17-oxo-DHA 과 함께 처리할 경우 Keap1 의 분해가 저해되는 것을 관찰함으로써, 실제로 17-oxo-DHA 는 autophagy 과정을 통한 Keap1 의 분해를 유도한다는 것을 확인하였다.

결론적으로, 친전자성 물질인 17-oxo-DHA 는 Keap1 의 cysteine 잔기의 변형을 통해 Keap1 의 autophagy 에 의한 분해를 유도함과 동시에 생체 내 방어기전을 유도하는 Nrf2 의 분해를 저해하여 활성화 시킴으로써, 세포 내 항산화 효소의 발현을 증진시킨다. 이상의 연구 결과를 통해 정상세포 내에서 17-oxo-DHA 의 보다 정확한 작용기전을 규명함으로써 17-oxo-DHA 가 산화적 스트레스에 의한 세포 손상을 방지하여 암을 비롯한 여러 질병의 발생을 예방하는 잠재적 후보물질임을 시사 하는 바이다.