약학석사학위논문

17-Oxo-Docosahexaenoic acid induces Nrf2-mediated expression of heme oxygenase-1 and NAD(P)H:quinone oxidoreductase in mouse skin

17-Oxo-Docosahexaenoic acid에 의한 마우스 피부에서 Nrf2 활성화 및 표적단백질 발현 유도

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Abstract

17-Oxo-Docosahexaenoic acid induces Nrf2-mediated expression of heme oxygenase-1 and NAD(P)H:quinone oxidoreductase in mouse skin

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Dietary omega-3 polyunsaturated fatty acids (PUFAs) have been shown to have therapeutic and preventive potential in the management of oxidative stress- and inflammation-associated ailments, such as diabetes mellitus, neurodegenerative disorders and various types of cancers. Docosahexaenoic acid (DHA), a representative omega-3 PUFA, is capable of activating multiple anti-oxidant and cytoprotective signaling pathways. Nrf2 plays a key role in cellular stress responses. In the present study, the effects of DHA on Nrf2-mediated expression of anti-oxidative enzymes were examined in mouse skin. Topical application of DHA significantly activated Nrf2 and upregulated expression of its target proteins, namely heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase (NQO-1). According to the recent studies, DHA undergoes metabolism to produce electrophilic oxo-derivatives that modulate several cellular redox signaling pathways more efficiently than the parent compound. One such metabolite is 17-oxo-DHA. Topical application of 17-oxo-DHA

markedly elevated the expression of Nrf2-mediated expression of HO-1 and NQO-1 in hairless mouse skin apparently by stimulating the degradation of cytosolic Keap1, a negative regulator of Nrf2. Notably, 17-oxo-DHA even at a lower dose (20 nmol), robustly upregulated the expression of aforementioned cytoprotective proteins as compared to DHA (10 μmol). In contrast to 17-oxo-DHA, 17-Hydroxy-DHA lacking the α,β-unsaturated carbonyl moiety was a much weaker inducer of Nrf2 activation and its target protein expression.

Keywords:

Mouse skin, Nrf2, Keap1, DHA, 17-oxo-DHA, cytoprotective proteins

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Introduction

Skin is constantly exposed to solar ultraviolet (UV) radiation. As a result of exposure to UV radiation, relatively large amounts of reactive oxygen species (ROS) are produced. When cellular anti-oxidant defense capacity is overwhelmed by ROS, the tissue rapidly confronts oxidative stress (Athar 2002, Bickers and Athar 2006). Various studies have demonstrated the deleterious effects of ROS-mediated oxidative stress in numerous maladies, such as inflammation, cancer, diabetes, etc (Maritim et al. 2003, Reuter et al. 2010, Surh et al. 2008). A wide array of natural or synthetic agents can prevent harmful effects of ROS by restoring normal redox homeostasis (Surh et al. 2008).

Cellular redox homeostasis is maintained by the constitutive induction of cytoprotective genes that encode distinct set of antioxidant and other cytoprotective proteins. These include heme oxygenase-1 (HO-1) and NAD(P)H: quinoneoxidoreductase-1 (NQO1) that are involved in cellular defense against oxidative stress(Nguyen et al. 2005). Thus, HO-1 and NQO-1 knockout mice were more prone to skin tumor development(Long et al. 2001, Was et al. 2011). The robust induction of aforementioned cytoprotective proteins is known to be mediated by nuclear factor erythroid 2-related factor 2 (Nrf2)(Surh et al. 2008).

Nrf2 is sequestered in the cytoplasm as an inactive complex with kelch-like ECH-associated protein 1 (Keap1) under unstressed conditions(Itoh et al. 2003). By interacting with Nrf2, Keap1 enhances degradation of Nrf2 rapidly with the help of Cul3 ubiquitin ligase complex. Once dissociated from Keap1, Nrf2 translocates into 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.

Health beneficial effects of omega-3 PUFAs have been corroborated by results from

various laboratory, population-based and clinical studies. A representative omega-3 fatty acids abundant in the fish oil is docosahexaenoic acid (DHA). Results from previous studies clearly showed that dietary supplementation of DHA alleviated symptoms of diverse pathological conditions linked to inflammation(Chapkin et al. 2009). Metabolism of DHA via cyclooxygenase (COX-2) and dehydrogenase-dependant pathways produces endogenous bioactive lipid mediators capable of triggering intracellular redox signaling pathways. An example is 17-oxo-DHA that has an electrophilic α,β-unsaturated carbonyl moiety(Groeger et al. 2010).

Owing to endogenous nature together with their potential to simultaneously stimulate multiple anti-inflammatory and cytoprotective pathways, DHA and 17-oxo-DHA hold great potential for preventing oxidative stress- as well as inflammation-associated skin diseases. In this study, I examined whether DHA and its endogenous electrophilic derivative 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*. Topical application of 17-Oxo-DHA enhanced the nuclear accumulation of Nrf2 while it decreased the steady state level of its inactive regulator Keap1. These results support our *in vitro* finding of the activation of Nrf2 cytoprotective signaling by 17-oxo-DHA in mouse epidermal JB6 cells. Notably, 17-oxo-DHA exerted much stronger effects than did DHA and its non-electrophilic precursor 17-OH-DHA on induction of Nrf2-mediated HO-1 expression.

Materials and Methods

Chemicals

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). Antibodies for NQO-1 and rabbit polyclonal HO-1 antibody were procured from Abcam (Cambridge, UK) and Enzo Life Sciences (Farmingdale, NY, USA) respectively. Primary antibodies for Lamin-B and α-Tubulin was the product of Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were obtained from Zymed Laboratories Inc. (San Francisco, CA, USA). The bicichoninic acid (BCA) protein assay reagent was supplied by Pierce Biotechnology (Rockfold, 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).

Animal treatment

Female HR-1 hairless mice (5-6 weeks age) were purchased from Sankyo Labo service Corporation, Inc. (SLC, Tokyo, Japan). Animals were kept in climate-controlled quarters

(24^0C 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) each was dissolved in 200 μl of acetone; These compounds were topically applied to the dorsal skin of mice for 2.5 or 5 h. Control animals were treated with vehicle only.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from skin tissue by using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the protocol supplied by manufacturer. One 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 used for further amplification in sequential reactions in the presence of HS Prime Taq Premix (Genetbio). The mRNA expression of *actin* (40 cycle of 94°C for 30 seconds, 49°C for 35 seconds, and 72°C for 30 seconds), *nrf2* (40 cycles of 94°C for 15 seconds, 51°C for 15 seconds, and 72°C for 30 seconds), *keap1* (35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds) and *nqo-1* (40 cycles of 94°C for 30 seconds, 50°C for 35 seconds, and 72°C for 30 seconds) was checked. After that, a final extension was performed for 7 minutes at 72°C. The primers used for each RT-PCR reactions are as follows: *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′; *nqo-1*, 5′-TCG GAG AAC TTT CAG TAC CC-3′ and 5′-TGC AGA GAG TAC ATG GAG CC-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 analyzed by 2% agarose gel electrophoresis, followed by staining with SYBR® green, and then photographed using fluorescence in LAS-4000.

Western blot analysis

For the preparation of the mouse epidermal protein extract, fat and dermis were taken off from the harvested skin samples 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 21,000 x *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 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 TBST buffer. The membranes were incubated for 4 h at room temperature with primary antibodies for HO-1 and overnight at 4^0C 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 afterwards.

Preparation of cytosolic and nuclear extracts

For homogenizing dorsal skin tissues of mice, 1 ml of buffer A [1 mM HEPES; 2 M KCl; 1 M $MgCl_2$; 0.1 M dithiothreitol (DTT); 0.1 M EDTA; 0.1 M phenylmethylsulfonylfluoride (PMSF)] was added. The homogenates was then centrifuged for 15 min at 18,000 x *g*; The supernatant was collected as cytosolic fraction and stored at -70^{0}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 M HEPES, 5 M NaCl, 0.1 M EDTA, 0.1 M DTT, 0.1 M PMSF, 100% glycerol], and subsequently centrifuged for 15 min at 21,000 x *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 fraction.

Immunofluorescence analysis (IF)

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 phosphate buffered saline (PBS) for 1 h. The tissues were then incubated with HO-1 primary antibody overnight. Alexa Fluor® conjugated secondary antibodies was used prior to mounting with 4',6-diamidino-2-phenylindole (DAPI) for the purpose of nuclear staining.

Statistical analysis

Results were expressed as the mean ± SEM 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.

Results

DHA elevated 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/Keap1 signaling in mouse skin, I initially examined the expression of Nrf2 protein by Western blot analysis. When shaven back of female C57BL/6 mice was treated topically with DHA (10 μmol), an elevated level of Nrf2 was observed compared to tissues left untreated (**Fig. 1A**). It was reported that Nrf2-mediated HO-1 expression was induced in BV-1 microglia in response to DHA treatment(Yang et al. 2013). Our results also displayed that the expression of HO-1 was elevated upon DHA application in mouse skin *in vivo*. Likewise, DHA treatment caused an increase in the expression of NQO-1, another Nrf2 target protein **(Fig. 1B)**.

DHA modulated the steady state levels of Nrf2 and Keap1 in HR-1 hairless mouse skin.

Another experiment was carried out to check the potential of DHA in triggering the Nrf2-dependent antioxidant response in hairless mouse skin. For that purpose, dorsal skin of female HR-1 hairless mice was treated topically with DHA (10 μmol) to measure the mRNA levels of *nrf2* and *keap1* in addition to measuring the expression of the Nrf2 target protein NQO-1 as well as total Nrf2 and Keap1. After 2.5 h of DHA application, RT-PCR analysis was performed to detect the mRNA expression of *nrf2*, *keap1* and *actin* (**Fig. 2A**). The mRNA expression levels of *nrf2* and *keap1* did not change in response to DHA

treatment which is in a good agreement with a previous finding(Yang et al. 2013). Next, the Nrf2 protein level in the epidermal tissue lysates was analyzed by Western blot analysis. As illustrated in **Fig. 2B**, the expression of Nrf2 protein significantly increased, whereas the expression of Keap1 in cytosolic fractions as well as in whole tissue lysates was decreased. In addition, RT-PCR analysis revealed increased mRNA expression of a representative Nrf2-regulated antioxidant gene, *nqo-1* in the mouse skin (**Fig. 2C**). These findings indicate that DHA stimulates Nrf2 target protein NQO-1 acting at a transcriptional level.

17-Oxo-DHA increased nuclear translocation of Nrf2 and decreased the steady state level of Keap1 in HR-1 hairless mouse skin.

Our *in vitro* data demonstrated that 17-oxo-DHA increased the nuclear translocation of Nrf2 and that the steady state level of Keap1 decreased in mouse epidermal JB6 cells stimulated with 17-oxo-DHA. As a consequence of Nrf2 stimulation, the upregulation of HO-1 and NQO-1 expression was seen in the same cultured epidermal cells. A similar Nrf2-dependent antioxidant response was observed in human macrophages treated with 17-oxo-DHA(Groeger A. L. et al. 2010). These *in vitro* results prompted me to assess the effects of 17-oxo-DHA on the activation of Nrf2 in mouse skin *in vivo*. After 2.5 h of 17-oxo-DHA (20 nmol) treatment onto the dorsal skin of female HR-1 hairless mice, the protein level of Nrf2 was checked by Western blot analysis. 17-Oxo-DHA treatment significantly increased nuclear accumulation of Nrf2 **(Fig. 4A)**. Nrf2 in the nucleus regulates expression of its target proteins, such as HO-1 and NQO-1(Kobayashi et al. 2006, Lee and Lee 2011). To confirm the expression of these proteins in skin tissues induced with 17-oxo-DHA, Western blot analysis was conducted. **Fig. 4B** illustrates that the protein

levels of HO-1 and NQO-1 were enhanced in 17-oxo-DHA-treated skin tissues. Cul3-mediated ubiquitination is switched from Nrf2 to Keap1 in response to electrophilic stimulus, which enhances the subsequent degradation of Keap1(Hong et al. 2005, Zhang D. D. et al. 2005). As shown here **(Fig. 4A),** topically applied 17-oxo-DHA also decreased the steady state level of Keap1.

Next, I also investigated the stimulation of Nrf2/Keap axis and its target proteins after 5 h of 17-oxo-DHA application. The mRNA expression levels of *nrf2* and *keap1* were examined by RT-PCR analysis. The mRNA expression of *nrf2* and *keap1* remained constant in skin tissues treated with 17-oxo-DHA **(Fig. 4C)**. In contrast, 17-oxo-DHA treatment markedly increased the protein level of Nrf2 **(Fig. 4D)**. These data clearly suggest that 17-oxo-DHA mediated the expression of Nrf2 by post-transcriptional mechanisms. In response to 17-oxo-DHA treatment, the level of HO-1 was also elevated to a greater extent than that achieved upon application of the same compound for 2.5 h **(Fig. 4D)**. The induction of HO-1 expression by 17-oxo-DHA was verified by immunofluorescence staining as well **(Fig. 4E)**.

17-Oxo-DHA at a lower dose enhanced the induction of Nrf2-regulated cytoprotective protein expression to a greater extent than DHA.

In the next experiment, the comparative effects of 17-oxo-DHA and its parent molecule DHA on the expression levels of HO-1 and NQO-1 protein were examined in skin tissues treated with each of these compounds. Notably, the expression of Nrf2-regulated cytoprotective proteins, namely HO-1 and NQO-1, was markedly increased even by a lower dose (20 nmol) of 17-oxo-DHA **(Fig. 5B)**. 17-Oxo-DHA more robustly promoted the

induction of Nrf2 than did DHA **(Fig. 5A)**. Taken together, these results strongly suggest that 17-oxo-DHA, as a bioactive metabolite of DHA, is a potent inducer of Nrf2 anti-oxidative response in mouse skin.

17-Oxo-DHA more robustly stimulates Nrf2/Keap1 signaling pathway and its target proteins than its non-electrophilic precursor 17-OH-DHA.

It is speculated that the electrophilic nature of 17-oxo-DHA confers this metabolite the potential to trigger Nrf2-mediated antioxidative response in mouse skin. To test this speculation, the protein levels of Nrf2 were compared between skin tissues stimulated with 17-oxo-DHA and its non-electrophilic precursor 17-OH-DHA at the same dose. As seen in **Fig. 6B**, 17-oxo-DHA more strongly induced nuclear translocation of Nrf2 than 17-OH-DHA. As expected, 17-oxo-DHA was more effective in promoting Keap1 degradation than 17-OH-DHA in mouse skin *in vivo* **(Fig. 6B, C)**. Likewise, the expression of HO-1 and NQO-1 induced by 17-OH-DHA in mouse skin was much less than that induced by 17-oxo-DHA **(Fig. 6C)**.

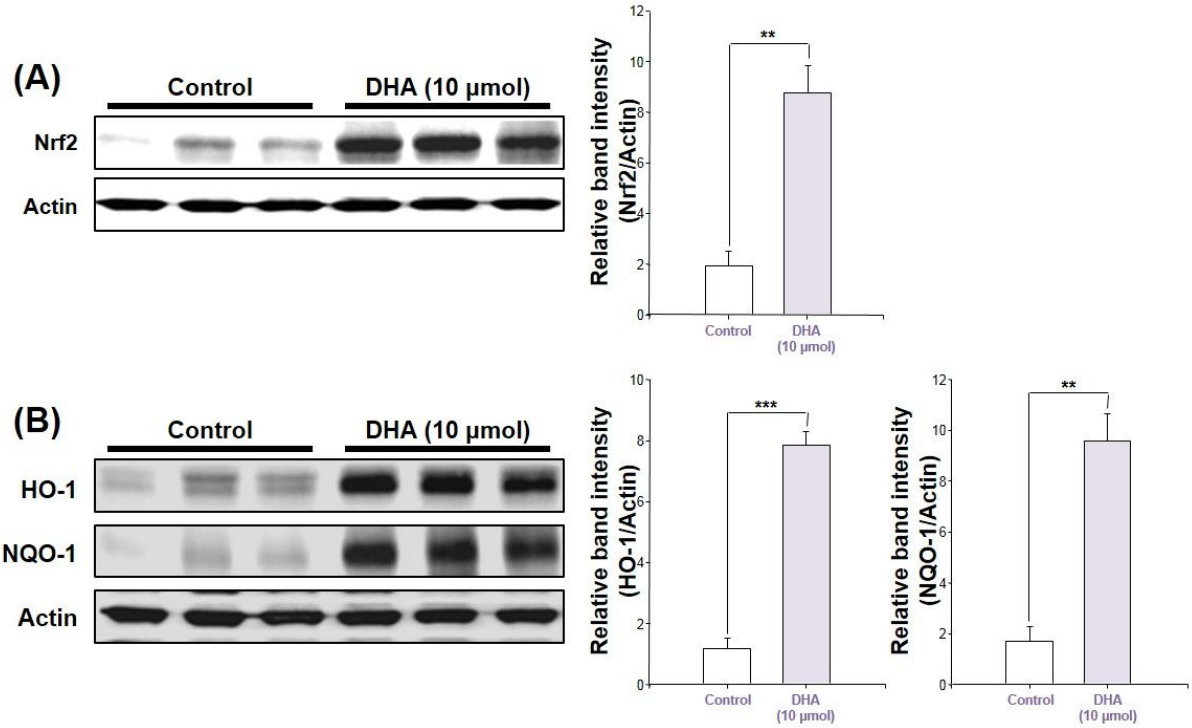


Figure 1. Effects of DHA on the expression of Nrf2 and its target proteins in mouse skin *in vivo*. Dorsal skin of shaven back of female C57BL/6 mice (n = 3 per group) was treated topically with 10 μmol of DHA. (A) After 4 h of DHA treatment, the Nrf2 protein level was examined in the epidermal tissue lysates by Western blot analysis. (B) The expression of Nrf2 target proteins, HO-1 and NQO-1, was also analyzed by Western blot analysis. Data are means ± SE. $^{**}p < 0.01$ and $^{***}p < 0.001$.

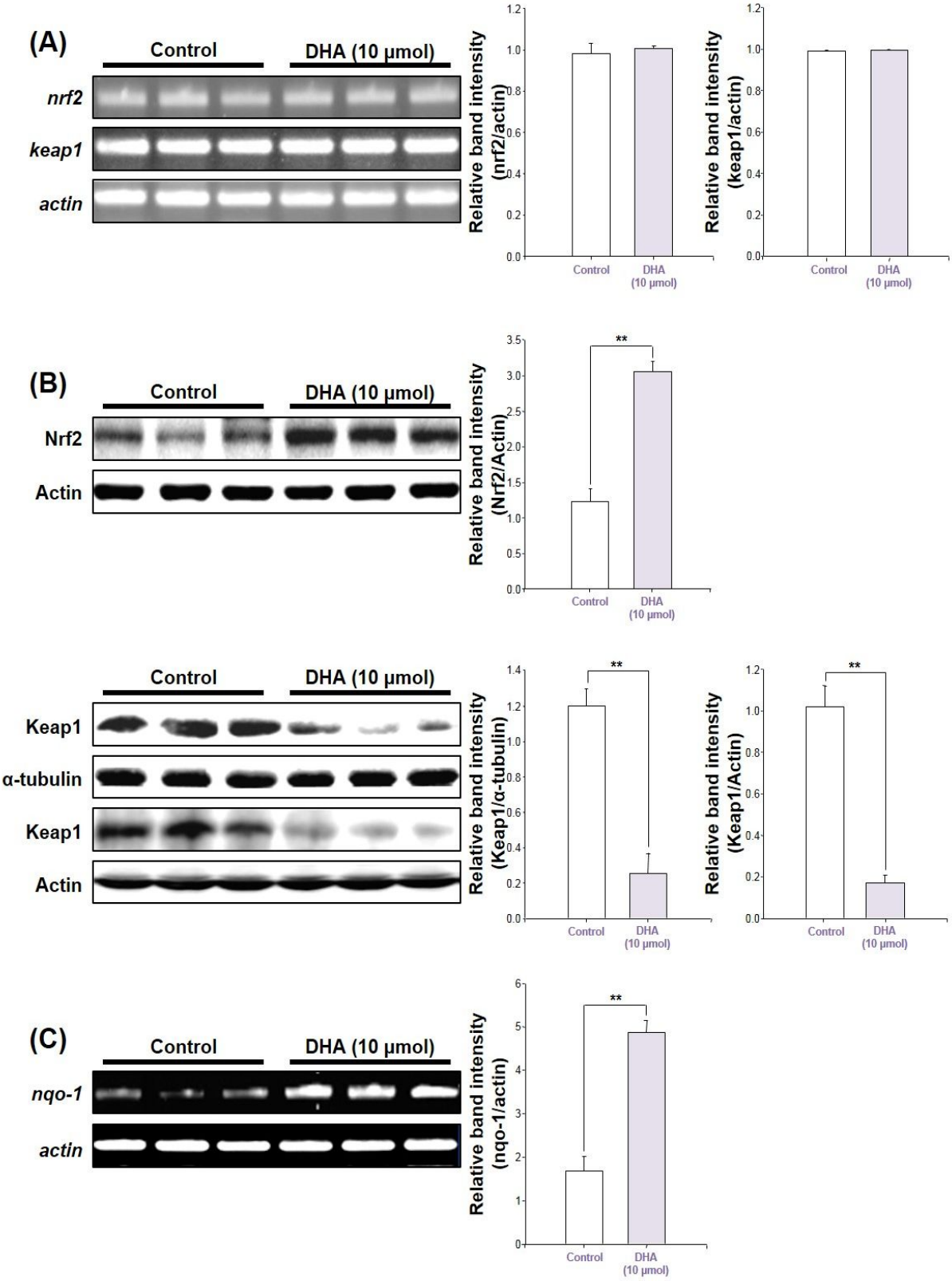

(A)
Control
DHA (10 μmol)
nrf2
keap1
actin
Relative band intensity (nrf2/actin)
Relative band intensity (keap1/actin)
Control
DHA (10 μmol)
(B)
Nrf2
Actin
Relative band intensity (Nrf2/Actin)
**
Keap1
α-tubulin
Keap1
Actin
Relative band intensity (Keap1/α-tubulin)
Relative band intensity (Keap1/Actin)
(C)
nqo-1
actin
Relative band intensity (nqo-1/actin)

Figure 2. Effects of DHA on the steady state levels of Nrf2 and Keap1 in HR-1 hairless mouse skin. Dorsal skin of female HR-1 hairless mice (*n* = 3 per group) was treated topically with DHA (10 μmol). (A) After 2.5 h of DHA application, RT-PCR analysis was performed to measure mRNA expression of *nrf2, keap1*, and *actin*. (B) The Nrf2 protein level in the epidermal tissue lysates was analyzed by Western blot analysis. The expression of Keap1 in cytosolic fractions and tissue lysates was also determined by Western blot analysis. (C) RT-PCR analysis was conducted to measure the mRNA levels of the Nrf2-regulated antioxidant gene, *nqo-1* in the mouse skin. Data are means ± SE. ***p* <0.01.

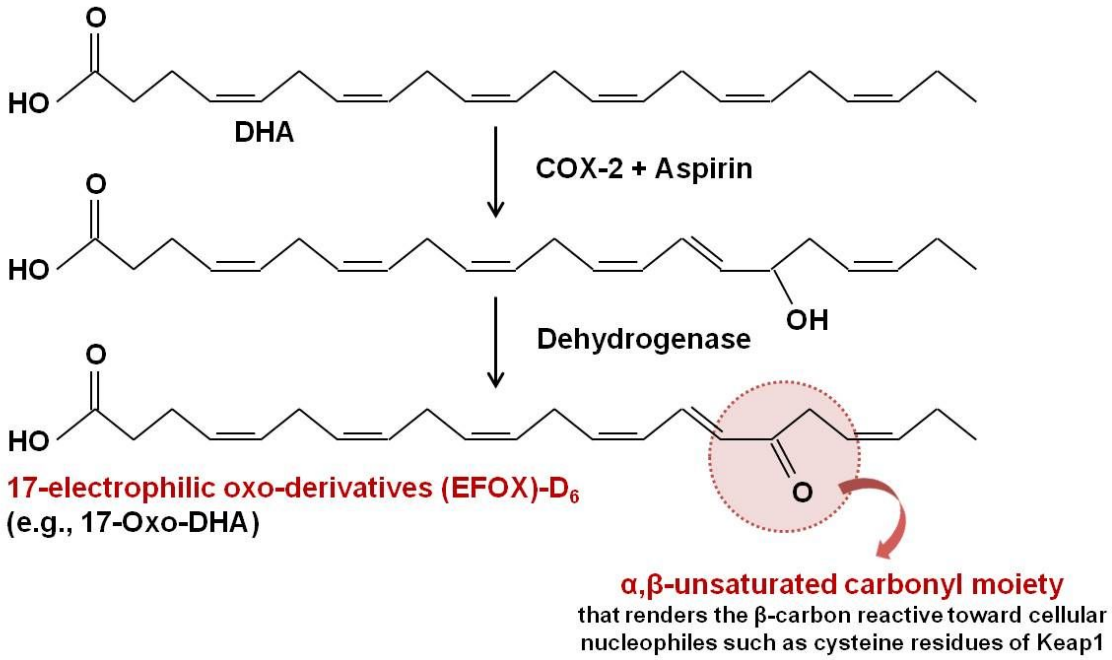
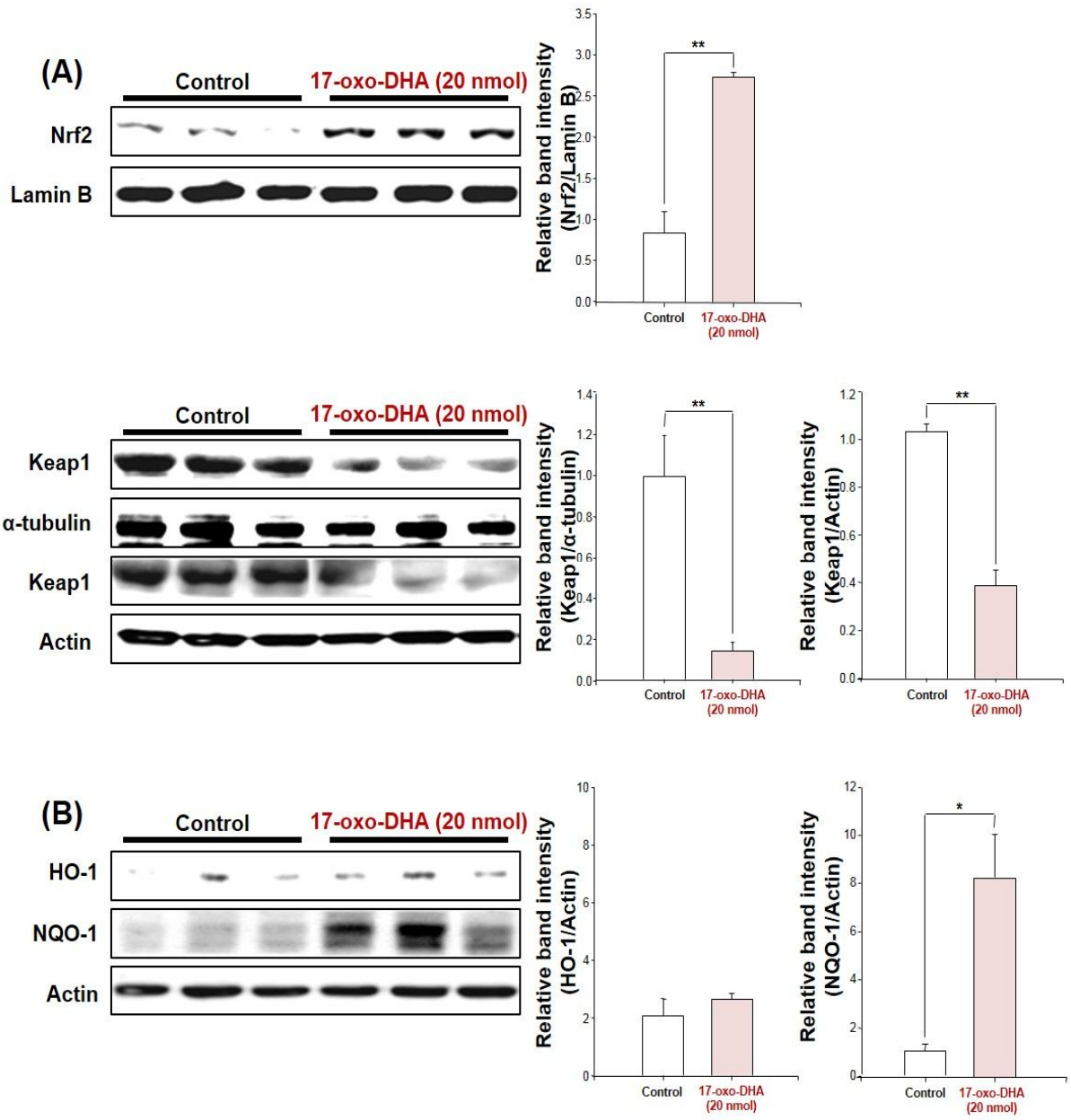


Figure 3. Metabolism of DHA to 17-oxo-DHA. In the presence of COX-2 and dehydrogenase, DHA is converted into 17-oxo-DHA, which is an electrophilic α,β-unsaturated ketone derivative. The α,β-unsaturated carbonyl moiety of 17-oxo-DHA is speculated to take part in the covalent interaction with nucleophilic residues of the target proteins.


(A)
Control
17-oxo-DHA (20 nmol)
Nrf2
Lamin B
Relative band intensity (Nrf2/Lamin B)
**
Control
17-oxo-DHA (20 nmol)
Keap1
α-tubulin
Keap1
Actin
Relative band intensity (Keap1/α-tubulin)
Relative band intensity (Keap1/Actin)
(B)
HO-1
NQO-1
Actin
Relative band intensity (HO-1/Actin)
Relative band intensity (NQO-1/Actin)
*

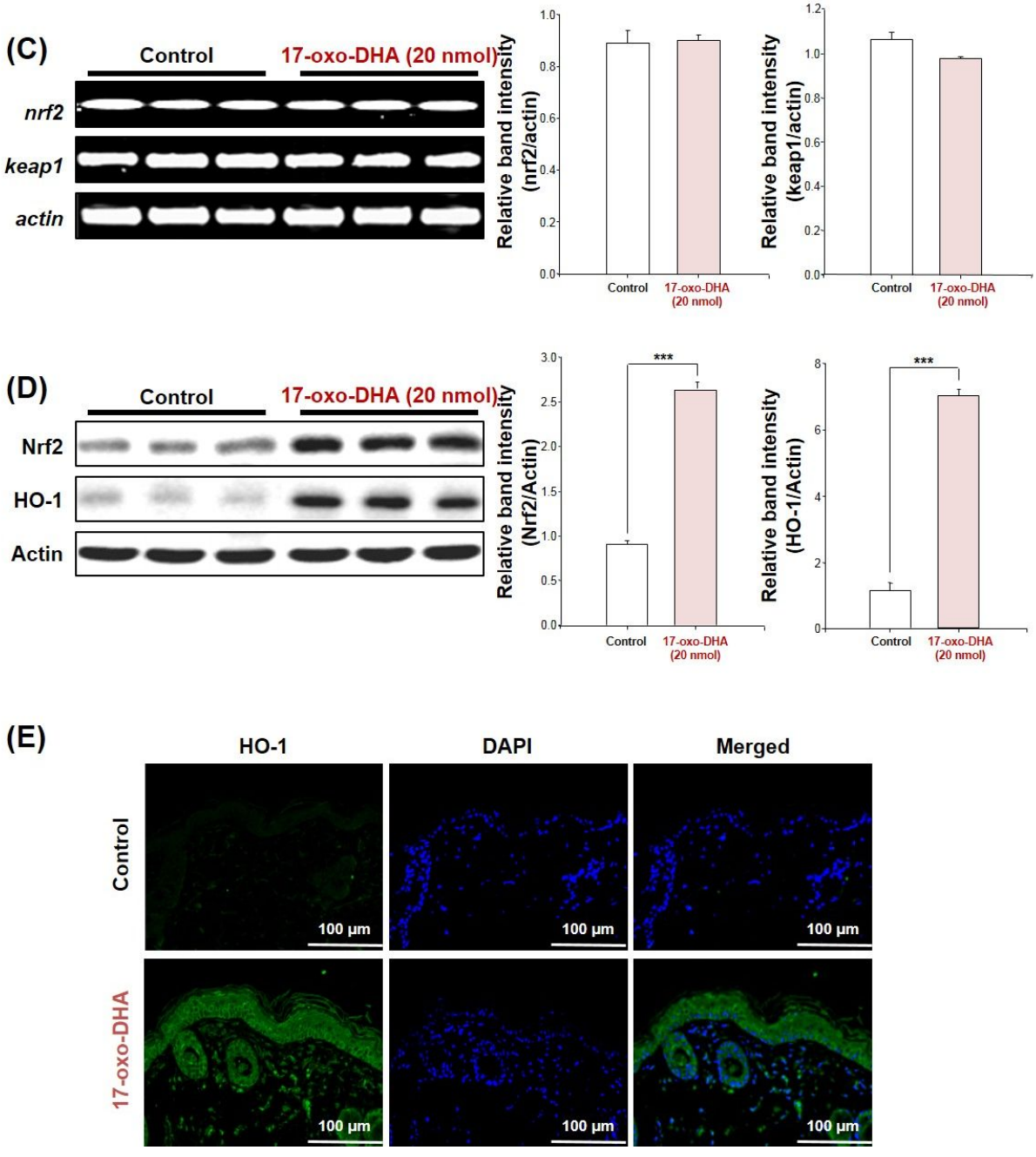


Figure 4. Effects of 17-oxo-DHA on the Nrf2/Keap1 signaling and target protein expression in mouse skin. Dorsal skin of female HR-1 hairless mice (*n* = 3 per group) was treated topically with 17-oxo-DHA (20 nmol) for 2.5 h (A, B) and 5 h (C, D,

E). (A) After 2.5 h of 17-oxo-DHA treatment, the nuclear Nrf2 and cytosolic Keap1 levels were assessed by Western blot analysis. The levels of Keap1 were also assessed in the whole lysates by Western blot analysis. (B) The expression of Nrf2 target proteins, HO-1 and NQO-1 was detected in the whole lysates 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 was determined in the whole lysates by Western blot analysis as well. (E) Induction of HO-1 expression was further clarified by performing immunofluorescence analysis. Data are means ± SE. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

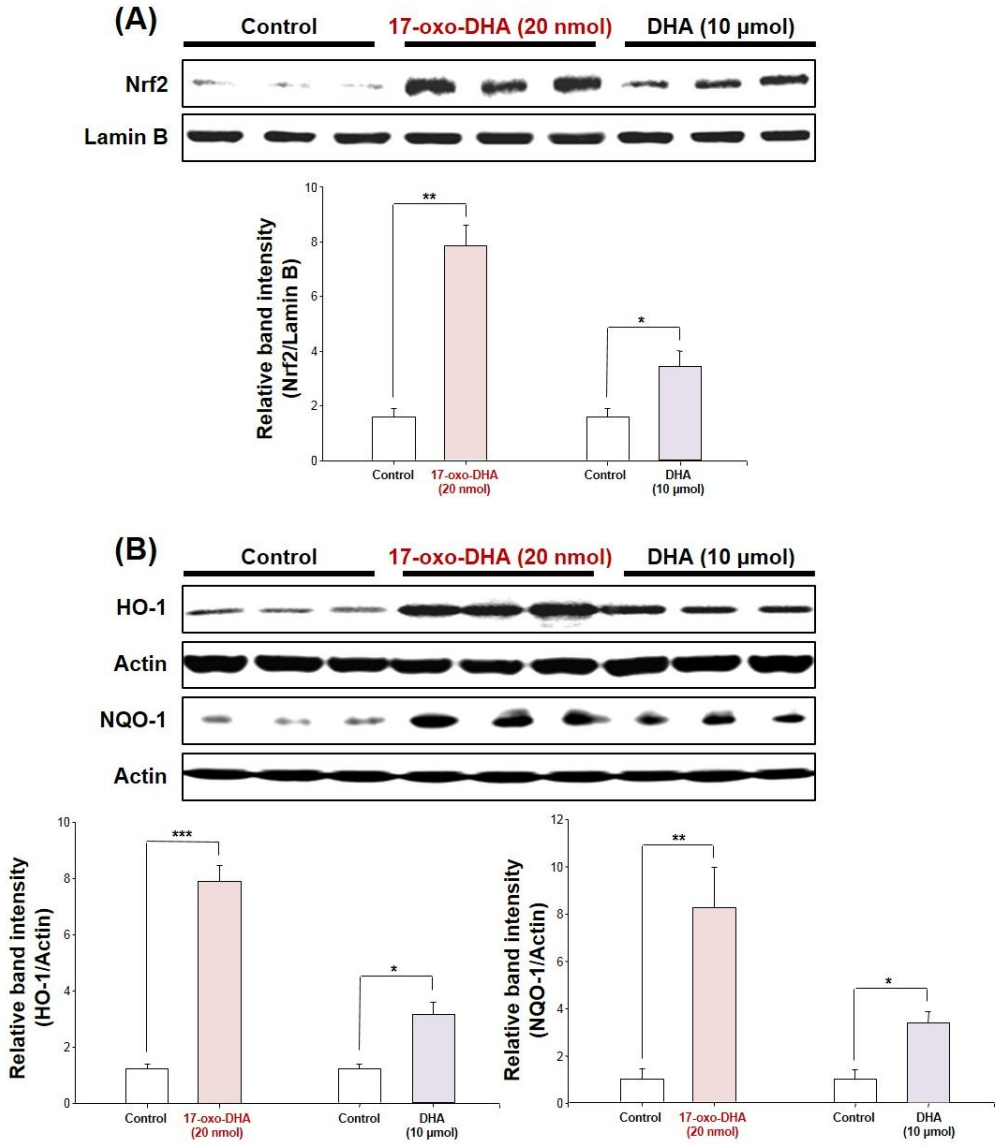


Figure 5. Comparative effects of 17-oxo-DHA and DHA on the nuclear translocation of Nrf2 and expression of HO-1 and NQO-1 in mouse skin. Dorsal skin of female HR-1 hairless mice (n = 3 per group) was treated topically with 17-oxo-DHA (20 nmol) or DHA (10 μmol). (A) After 5 h of topical application, the protein levels of Nrf2 were checked by Western blot analysis in the nuclear fraction. (B) The expression of Nrf2-regulated cytoprotective proteins was also determined in whole tissue

lysates by Western blot analysis. Data are means ± SE. *p <0.05, **p <0.01, and ***p <0.001.

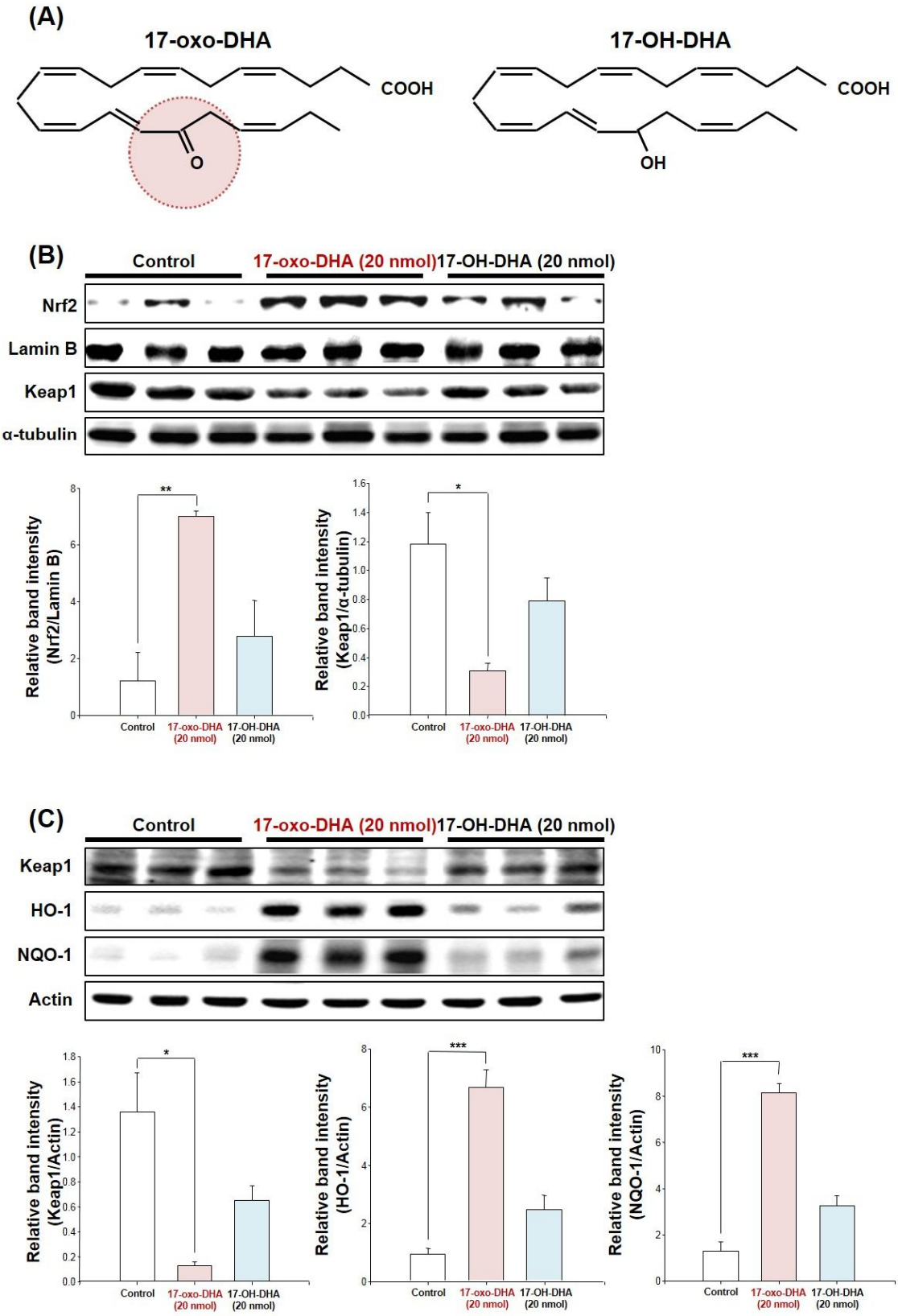

(A)
17-oxo-DHA
COOH
O
17-OH-DHA
COOH
OH
(B)
Control
17-oxo-DHA (20 nmol)
17-OH-DHA (20 nmol)
Nrf2
Lamin B
Keap1
α-tubulin
Relative band intensity (Nrf2/Lamin B)
Relative band intensity (Keap1/α-tubulin)
Control
17-oxo-DHA (20 nmol)
17-OH-DHA (20 nmol)
(C)
Control
17-oxo-DHA (20 nmol)
17-OH-DHA (20 nmol)
Keap1
HO-1
NQO-1
Actin
Relative band intensity (Keap1/Actin)
Relative band intensity (HO-1/Actin)
Relative band intensity (NQO-1/Actin)

Figure 6. 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) By the action of dehydrogenase, 17-oxo-DHA is generated from 17-OH-DHA, a hydroxylated metabolite of DHA. 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 analyzed by Western blot analysis. (C) The expression of Keap1, HO-1 and NQO-1 was also checked in the whole tissue lysates by Western blot analysis. Data are means ± SE. *p <0.05, **p <0.01, and ***p <0.001.

Discussion

An array of environmental chemical and physical agents exerts adverse effects through the generation of ROS. ROS-mediated oxidative stress is capable of fostering the development of various pathological conditions mainly by overwhelming skin homeostatic defenses. In response to oxidative damage resulting from excessive ROS production, the expression of Nrf2-regulated cytoprotective proteins including HO-1 and NQO-1 is often upregulated. In various cells, the induction of HO-1 provides protective effects against oxidative stress(Abraham and Kappas 2005). Therefore, dysfunction of aforesaid cytoprotective mechanism in skin has been associated with severe redox dermal stress and photocarcinogenesis. In line with this notion, Nrf2 knockout mice were shown to be more sensitive to chemically induced skin carcinogenesis(Xu et al. 2006). In a similar way, the severity of chemically induced skin tumorigenesis was higher in NQO-1 null mice than in that mice expressing wild-type NQO-1(Long et al. 2001).

Many previous studies have focused on broad spectrum of cytoprotective molecules that can obviate the detrimental effects of oxidative damage(Yang et al. 2013). A wide variety of anti-oxidative agents which are constituents of our daily diet have been demonstrated to combat skin cancer triggered by UVB irradiation(Chapkin et al. 2009). In recent years, dietary omega-3 PUFAs have been investigated for a wide range of health beneficial effects, such as anti-oxidative, anti-inflammatory, and chemopreventive activities(MacLean et al. 2006). A growing body of evidence supports that DHA is responsible for these salutary effects. DHA, a representative omega-3 PUFA, modulates multiple signaling pathways including one mediated by Nrf2/Keap1(Yang et al. 2013). In

this context, anti-oxidant capability of DHA to cope with UVB-promoted oxidative stress has been reported(Rahman et al. 2011).

It has been reported that transient production of ROS is induced in cells supplemented with DHA(Aires et al. 2007)(Lu et al. 2010). Relevant to this aspect, several pathways including cysteine oxidation, Zn release, and phosphorylation by PKC, PI3K and ERK mediate ROS-induced Keap1 modification(D'Autreaux and Toledano 2007). The redox sensitive Nrf2/Keap1 system regulates the induction of HO-1(Kobayashi et al. 2006). HO-1 expression is also triggered in response to DHA treatment in EA.hy926 cells(Yang et al. 2013). In light of these findings, it is speculated that DHA-induced ROS production might trigger Nrf2/Keap1 signaling by which the expression of HO-1 and NQO-1 is upregulated. In view of this concept exhibiting the anti-oxidative role of DHA via stimulation of Nrf2, I sought to investigate whether topical application of DHA could produce similar anti-oxidative response in mouse skin *in vivo*; I found that topically applied DHA increased nuclear translocation of Nrf2, thereby leading to HO-1 induction and that the DHA also decreased the steady state level of total and cytosolic Keap1 protein. Concisely, this finding suggests that DHA promoted degradation of Keap1, which facilitated the dissociation of Nrf2. Subsequently, translocation of Nrf2 into nucleus up-regulates expression of anti-oxidative proteins, namely HO-1 and NQO-1.

It has been reported that electrophilic oxygenated derivatives of omega-3 PUFAs are active mediators responsible for their observed beneficial effects(Cipollina 2015). These electrophilic oxo-derivatives are usually produced by cells under oxidative stress and inflammation(Cipollina 2015). The intracellular levels of these species are also elevated with aspiring-triggered COX-2 activity(Freeman 2012). These electrophilic signaling

species modulate intracellular redox signaling pathways by covalently binding to nucleophilic residues present in target proteins. This leads to the stimulation of a complex cascade of cytoprotective events including anti-oxidative pathways(Gao et al. 2007, Groeger et al. 2010). A recent study has demonstrated that the conversion of DHA into bioactive oxygenated derivatives takes place during oxidation reactions either through enzymatic or non-enzymatic mechanisms. An example of DHA-derived electrophilic metabolite is 17-oxo-DHA that has an α,β-unsaturated ketone moiety(Groeger et al. 2010).

It is important to note that the Nrf2-induced cytoprotective pathway, the heat shock response, the NF-κB inflammatory pathway contribute to the bioactivities of omega-3 PUFAs and their electrophilic derivatives such as, 13- and 17-oxo-DHA, and A4/J4-neuroprostanes(Cipollina et al. 2014, Lefils-Lacourtablaise et al. 2013, Musiek et al. 2008a). Of note, several electrophilic metabolites derived from omega-3 PUFAs have been shown to promote Nrf2 nuclear accumulation followed by induction of its target gene expression in different experimental models(Cipollina et al. 2014, Gao et al. 2007, Zhang M. et al. 2014). These electrophilic signaling species specifically interact with cysteine residues of Keap1 and enhanced its subsequent degradation, resulting in activation of Nrf2(Freeman 2012, Kansanen et al. 2009). Results from *in vitro* displayed the enhanced Nrf2/Keap1 anti-oxidant response in mouse epidermal JB6 cells subjected to 17-oxo-DHA treatment. Consistent with *in vitro* findings, topical application of 17-oxo-DHA promoted the degradation of Keap1, whereas it increased the protein level of Nrf2 in mouse skin *in vivo*. Notably, 17-oxo-DHA at a relatively low dose, as compared to other activators of Nrf2, stimulates the Nrf2/Keap1 anti-oxidative response(Cipollina et al. 2014, Gao et al. 2007, Musiek et al. 2008b, Schopfer et al. 2011). Thus, 17-oxo-DHA at a dose as low as 20nmol,

activated Nrf2-mediated anti-oxidant signaling response to a greater extent than DHA.

In contrast to 17-oxo-DHA, its non-electrophilic precursor 17-OH-DHA induced much weaker induction of Nrf2 activation and its target protein expression. 17-hydroxy-DHA may undergo oxidation to 17-oxo-DHA when treated to mouse skin, possibly by epidermal dehydrogenase activity, and this may account for its ability to activate Nrf2 signaling. Briefly, results from these experiments substantiated our hypothesis that oxygenation of DHA is responsible for, at least in part, its biological activity in mouse skin.

In conclusion, 17-oxo-DHA robustly induces Nrf2/Keap1 anti-oxidative response in mouse skin *in vivo***.** This activity of 17-oxo-DHA is attributable to its electrophilic nature that makes this molecule more reactive towards regulatory proteins of antioxidant signaling pathway, such as Keap1. Thus, 17-oxo-DHA holds great potential in the management of skin diseases characterized by persistent oxidative stress or inflammation.

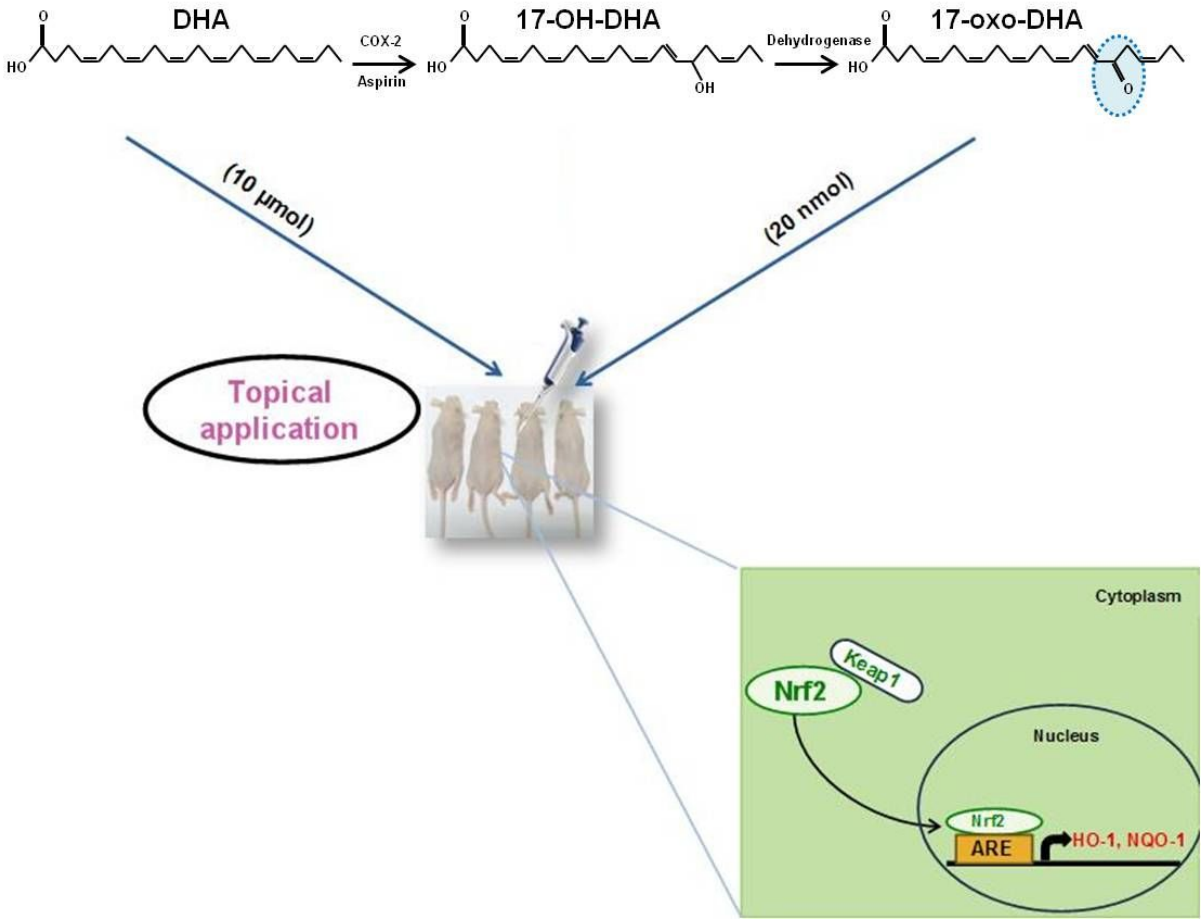


Figure 7. Schematic illustration of Nrf2 activation and antioxidant gene expression by DHA and its electrophilic metabolite 17-oxo-DHA

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