藥學碩士學位論文

Protective Effects of Docosahexaenoic Acid against UVB-induced Mouse Skin Carcinogenesis

UVB 에 의해 유도된 마우스 피부발암과정에서 Docosahexaenoic Acid 의 암예방 효과

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Abstract

Protective Effects of Docosahexaenoic Acid against UVB-induced Mouse Skin Carcinogenesis

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Exposure to solar radiation, especially ultraviolet B (UVB), is the most prevalent environmental carcinogen that increases the risk of skin cancer. Oxidative stress and persistent inflammation are the key pathologic events in UVB-induced skin photocarcinogenesis. Signal transducer and activator of transcription-3 (STAT3) 3, one of the major redox-sensitive transcription factors, plays an essential role in the pathogenesis of inflammation and subsequent development of skin cancer. Omega-3 polyunsaturated fatty acids

(ω-3 PUFAs), which occur at high levels in some fish oils, are known to possess radical scavenging activity and increase host immunoresponsiveness. The present study was aimed at evaluating the anti-inflammatory and anti-tumor promoting activity of docosahexaenoic acid (DHA), a prototypic ω-3 PUFA, in mouse skin irradiated with UVB. Topical application of DHA (2.5 and 10 μmol) prior to UVB irradiation (180 mJ/cm^2) diminished epidermal hyperplasia and formation of 4-hydroxynonenal modified protein and malondialdehyde, the biochemical hallmarks of lipid peroxidation, in HR-1 hairless mouse skin. Pretreatment with DHA inhibited UVB-induced phosphorylation of STAT3 (Tyr^{705}) and expression of its target gene, *c-myc* in the mouse skin. Moreover, repeated DHA pretreatment until the termination of the experiment at the 23rd week reduced the incidence and the multiplicity of papillomas and squamous cell carcinomas in UVB-induced mouse skin. DHA down-regulates STAT3 activation and c-Myc expression in papillomas as compared to ultraviolet radiation alone. Furthermore, topically applied DHA induced the expression of antioxidant enzymes, heme oxygenase-1 and NAD(P)H:quinone oxidoreductase 1 in the mouse skin. Taken together, DHA protected against UVB-induced mouse skin tumor development by blocking STAT3 activation and enhancing inducing antioxidant enzyme expression, suggesting that this ω-3 PUFA has potential for use to ameliorate UVB-induced skin tissue damage and related abnormal disorders including

photocarcinogenesis.

Keywords: UVB, Skin carcinogenesis, STAT3, DHA, Antioxidant enzymes

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Introduction

Ultraviolet (UV) radiation is an important environmental factor responsible for the pathogenesis of skin aging and photocarcinogenesis. According to the *World Cancer Report*, skin cancer constitutes approximately 30% of all newly diagnosed cancers in the world. Exposure to UVB radiation is the major cause of 90% of skin cancers. Irradiation with UVB induces oxidative stress and inflammatory tissue damage, thereby causing squamous and basal cell carcinomas (Afaq et al. 2005, Halliday 2005). UVB exerts its detrimental effect mainly through direct DNA damage and the generation of reactive oxygen species (ROS), which cause oxidative or covalent modification of cellular macromolecules, such as lipids, proteins and nucleic acids. For example, the UVB irradiation increases the accumulation of lipid peroxidation products, such as 4-hydroxynonenal (4HNE) and malondialdehyde (MDA) (Oberley et al. 2004, Petersen and Doorn 2004, Tyrrell 1995). Moreover, UVB-induced generation of ROS amplify intracellular signaling pathways comprising various kinases and transcription factors (Bachelor et al. 2005, Bode and Dong 2003, Dickinson et al. 2011). One of the UVB-responsive transcription factors is signal transducer and activator of transcription-3 (STAT3), which promotes the transcriptional

upregulation of genes involved in cell proliferation and tissue inflammation (Kundu and Surh 2012). The activation of STAT3 is dependent on the phosphorylation of tyrosine-705 (Y705) residue and serine-727 (S727) residue. Upon phosphorylation of these serine and tyrosine residues, STAT3 is dimmerized and translocated into the nucleus, where its binds to the promoter regions of genes encoding cell survival proteins, such as cyclins, c-Myc, survivin, Bcl-2, Bcl-xl (Aaronson and Horvath 2002, Turkson et al. 1999, Zhang et al. 2001). Aberrant activation of STAT3 has been implicated in UVB-induced skin carcinogenesis (Kim et al. 2009). Thus, the normalization of inappropriately amplified STAT3 signaling cascade might be a rational approach for chemoprevention of skin carcinogenesis.

Since UVB-induced ROS trigger skin photo-inflammation and photocarcinogenesis, the mitigation of oxidative stress can protect cellular macromolecules from oxidative damage or covalent modification. A battery of endogenous antioxidants maintains the cellular redox balance. These include a series of antioxidants and detoxification enzymes, collectively called cytoprotective proteins, are involved in the hermetic control of cellular redox status (Surh et al. 2008). For instance, hemeoxygenase-1 (HO-1) and NAD(P)H quinoneoxidoreductase-1 (NQO1) have been reported to protect skin from oxidative and inflammatory tissue injury and carcinogenesis. A recent study demonstrated that mice harboring wild type HO-1 are less

susceptible to chemical carcinogen-induced skin inflammation and tumor formation in the early stage of papillomagenesis as compared to HO-1 knockout mice (Was et al. 2011). Likewise, the NQO1-null mice are more susceptible to chemically induced skin tumor development (Long et al. 2000, Long et al. 2001). Thus, the fortification of the expression and/or activity of HO-1 and NQO1 is a practical approach to prevent skin carcinogenesis. The promoter regions of genes encoding HO-1 and NQO1 harbors *cis*-acting regulatory sequences, known as antioxidant response element (ARE) or electrophile response element (EpRE) (Surh et al. 2008). Mild oxidative and/or electrophilic stress activates a redox-sensitive transcription factor nuclear factor-erythroid related factor-2 (Nrf2), which by interacting with the ARE sequences upregulate the expression of cytoprotctive proteins, such as HO-1 and NQO1 (Surh et al. 2008). Thus, one of the potential strategies to prevent skin cancer is to enhance the Nrf2-mediated induction of cytoprotective proteins.

A wide variety of dietary antioxidant and anti-inflammatory substances have been reported to prevent skin cancer (Gupta and Mukhtar 2001). A class of promising dietary chemopreventive substances is ω-3 polyunsaturated fatty acids (PUFAs) present in fish oil and plant-based diets including flaxseed oil. Docosahexaenoic acid (DHA) (**Fig. 1**), a representative ω-3 PUFAs, has been extensively investigated for health beneficial effects, such as antioxidative,

anti-inflammatory, neuroprotective and chemopreventive activities (Jho et al. 2004, Rahman et al. 2011). The effects of DHA on UVB-induced skin photocarcinogenesis and its underlying mechanisms have not been investigated yet. Here, we report that topical application of DHA inhibits UVB-induced skin papillomagenesis, partly, by blocking STAT3 signaling and inducing Nrf2-mediated expression of HO-1 and NQO1.

Materials and Methods

Materials

DHA (purity > 98%) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Primary antibodies for pSTAT3 and STAT3 were procured from Cell Signaling Technology (Beverly, MA, USA). Antibodies for c-Myc, Nrf2 and HO-1 were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies for Lamin-B was purchased from BD Biosciences (San Jose, CA, USA). Antibodies of 4-HNE-modified proteins and MDA were obtained from Japan Institute for the Control of Aging (Shizuoka, Japan). Anti-actin antibody was obtained from Sigma Chemical Company (St. Louis, MO, USA). Antibody against NQO1 was obtained from Abcam (Cambridge, UK). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were obtained from Zymed Laboratories Inc. (San Francisco, CA, USA). Enhanced chemiluminescent (ECL) detection kit and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals used were in the purest form available commercially.

Animal treatment

Female HR-1 hairless mice (6-7 weeks age) were supplied from Sankyo Laboservice Corporation, Inc. (SLC, Tokyo, Japan). Animals were housed in climate-controlled quarters (24 ± 1^{0}C at 50% humidity) with a 12-h light/12-h dark cycle. DHA (2.5 and 10 µmol) was dissolved in 200 µl of acetone and applied topically to the dorsal skin 40 min before exposure to UVB (180 mJ/cm^2) radiation.

Source of UVB radiation

The UVB radiation was a 5 × 8 Watt tube, which emits an energy spectrum with high fluency in the UVB region (with a peak at 312 nm). A Biolink BLX-312 UV crosslinker (Vilbert Lourmat, Marne-la-Valée, France) was used in the present study to irradiate mouse skin.

Western blot analysis

Dorsal skin of HR-1 hairless mice were treated with DHA (2.5 or 10 µmol/mouse) 40 min before exposure to UVB (180 mJ/cm^2) and sacrificed by cervical dislocation 2.5 h later. Control animals were treated with vehicle only. For the preparation of mouse epidermal protein extract, fat and dermis were removed from the harvested skin samples kept on ice and the fat-free epidermis was immediately placed in liquid nitrogen and pulverized in mortar. The pulverized skin was homogenized on ice for 20 s with a polytron

tissue homogenizer and lysed in 1 mL ice-cold lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl pH 7.4, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4 and protease inhibitor cocktail tablet). Lysates were centrifuged at 14,800 *g* for 15 min. The supernatant was collected and total protein concentration was quantified by using the bicinchoninic acid (BCA) protein assay kit. Cell lysates (30 µg protein) were boiled in sodium dodecyl sulfate (SDS) sample loading buffer for 5 min before electrophoresis on 10-12% SDS-polyacrylamide gel. After transfer to polyvinylidene difluoride (PVDF) membrane, the blots were blocked with 5% fat-free dry milk-PBST (Phosphate-buffer saline containing 0.1% Tween 20) or 1% bovine serum albumin (BSA) in TBST (Tris-buffer saline containing 0.1% Tween 20) for 1 h at room temperature and then washed with PBST or TBST buffer. The membranes were incubated for 2 h at room temperature with 1:1000 dilutions of primary antibodies for actin and for 12 h at 4 ^{0}C with 1:1000 dilutions of primary antibodies for 4-HNE modified protein, c-Myc, Nrf2, NQO-1, Lamin B, STAT3 and phosho-STAT3. Blots were washed three times with PBST or TBST at 5 min intervals followed by incubation with 1:5000 dilution of respective horseradish peroxidase conjugated secondary antibodies (rabbit, goat or mouse) in 3% fat-free dry milk-TBST for 1 h at room temperature. The blots were rinsed again three times with PBST or TBST. The immunoblots were visualized with an ECL detection kit

according to the manufacturer's instructions.

Histological analysis

Sections of harvested mouse skin were washed with phosphate-buffered saline (PBS) and fixed with 10% buffered formalin and embedded in paraffin. Each section (4 μm) was stained with hematoxylin and eosin (H&E). The H&E stained sections were examined under light microscope to detect the presence of lesions.

Immunohistochemical analysis

Mouse skin irradiated with UVB in presence or absence of DHA were subjected to immunohistochemical analysis for detecting the expression of 4-HNE modified protein, MDA and phospho-STAT3. Four-micrometer sections of 10% formalin-fixed, paraffin-embedded tissues were cut on salinized glass slides and deparaffinized three times with xylene, and rehydrated through graded alcohol bath. The deparaffinized sections were heated with microwave and boiled twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish non-specific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. For the detection of respective protein expression, slides were incubated with affinity purified rabbit polyclonal anti-4-HNE, anti-

MDA or anti-pSTAT3 (Y705) (1:50) at room temperature for 40 min in Tris-buffered saline containing 0.05% Tween 20 and then developed using anti-rabbit HRP EnVision^{TM} System (Dako, Glostrup, Denmark). The peroxidase binding sites were detected by staining with 3,3'-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark). Finally, counterstaining was performed using Mayer's hematoxylin.

Reverse transcriptase polymerase chain reaction

Total RNA was isolated from skin tissue by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. To generate the cDNA from RNA, 1 μg of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase (Promega) for 50 min at 42℃ and again for 15 min at 72℃. One microliter of cDNA was amplified in sequential reactions by using Maxime PCR PreMix Kit (iNtRON Biotechnology). For detection of HO-1 mRNA, 20 cycles of 94℃ for 30 seconds, 53℃ for 35 seconds, and 72℃ for 30 seconds were conducted; for quantitation of actin mRNA, 20 cycle of 94℃ for 30 seconds, 59℃ for 35 seconds, and 72℃ for 30 seconds were conducted. These PCR cycles were followed by a final extension for 7 minutes at 72℃. The primers used for each RT-PCR reactions are as follows: *HO-1*, 5′-TAC ACA TCC AAG CCG AGA AT-3′ and 5′-GTT CCT CTG

TCA GCA TCA CC-3′; *Nrf2,* 5′-TGC CCC TCA TCA GGC CCA GT-3′ and 5′-GCT CGG CTG GGA CTC GTG TT-3′; *NQO-1*, 5′-GAG GAC CTC CTT CAA CTA TG-3′ and 5′-CCTTTG TCA TAC ATG GCA GC-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). Amplification products were analyzed by 2.0% agarose gel electrophoresis, followed by staining with ethidium bromide, and then photographed under UV light.

Preparation of cytosolic and nuclear extracts

The cytosolic and nuclear extract from mouse skin was prepared as described previously. In brief, scraped dorsal skin of mice was homogenized in 800 μl of hypotonic buffer A [10 mM HEPES, pH 7.8; 10 mM KCl; 2 mM $MgCl_{2;}$ 1 mM DTT; 0.1 mM EDTA; 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. To the homogenates was added 80 μl of 10% Nonidet P-40 (NP-40) solution, and the mixture was then centrifuged for 2 min at 14000 *g*. The supernatant was collected as cytosolic fraction. The precipitated nuclei were washed once with 500 μL of buffer A plus 40 μl of 10% NP-40, centrifuged, resuspended in 200 μl of buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 20% glycerol] and centrifuged for 5 min at 14,800*g*. The supernatant containing nuclear

proteins was collected and stored at -70°C after determination of protein concentrations.

Statistical analysis

Values were expressed as the mean ± SEM of at least three independent experiments. Statistical significance was determined by Student's *t* test and a *p*-value of less than 0.05 was considered to be statistically significant.

Results

DHA attenuates UVB-induced lipid peroxidation and skin hyperplasia in female HR-1 mouse skin in vivo

UVB irradiation is known to produce ROIs, which leads to lipid peroxidation (Halliday 2005). Multiple lines of evidence suggest that UVB-induced generation of ROS, such as superoxide anion, hydroxyl radical and hydrogen peroxide, is responsible for skin hyperplasia (Tyrrell 1995). The 4-HNE and MDA are representative lipid peroxidation products (Oberley et al. 2004, Petersen and Doorn 2004). Immunohistochemical analysis of mouse skin irradiated with UVB in presence or absence of DHA (2.5 or 10 μmol) revealed that pretreatment with DHA attenuated UVB-induced skin hyperplasia (**Fig. 2A**, upper panel) and the levels of MDA (**Fig. 2A,** lower panel). DHA pretreatment also inhibited UVB-induced accumulation of 4-HNE-modified proteins in mouse epidermis (**Fig. 2B**).

DHA inhibits UVB-induced activation of STAT3 and expression of its target gene, c-myc in hairless mouse skin

Aberrant activation of STAT3 signaling has been implicated in skin carcinogenesis (Kim et al. 2009). It has been reported that exposure to UVB

radiation activates STAT3 via phosphorylation at tyrosine-705 residue in SKH1 hairless mouse skin (Ahsan et al. 2005). We, therefore, examined the effect of DHA on UVB-induced activation of STAT3 and the expression of its target gene c-Myc. As show in **Fig. 3A**, irradiation of HR-1 hairless mouse skin with UVB (180 mJ/cm^2) increased the phosphorylation of STAT3 at tyrosine-705 residue, which was blunted by pretreatment with DHA (2.5 or 10 μmol) in a dose-dependent manner. The inhibitory effect of DHA on UVB-induced STAT3 (Y705) phosphorylation was verified by immunohistochemical analysis. As shown in **Fig. 3B**, UVB irradiation markedly increased the expression of pSTAT3(Y705) appeared as brown color staining. Pretreatment with DHA attenuated UVB-induced expression of pSTAT3(Y705), while treatment with DHA alone maintained the pSTAT3(Y705) level comparable to that of control. Moreover, UVB irradiation elevated the mRNA expression of a STAT3 target gene, *c-Myc*, which was significantly diminished by pretreatment with DHA (**Fig. 3C**).

DHA inhibits UVB-induced papillomagenesis in HR-1 hairless mouse skin

Since DHA attenuated UVB-induced lipid peroxidation, skin hyperplasia and STAT3 activation, we examined the effect of repeated application of DHA on papillomagenesis in a UVB-induced skin carcinogenesis model. At the termination of experiment after 23 weeks, representative photographs of

mice from indicated treatment groups were taken. **Fig. 4A** shows that UVB irradiation alone induced skin papilloma formation, which was diminished by DHA pretreatment. We monitored the body weight of each mouse every week until termination of the experiment. The body weight change of UVB-treated mice was decreased compared with control group. However, administration of DHA prevented UVB-induced body weight loss **(Fig. 4B)**. Animals irradiated with UVB alone developed skin papillomas at 8th week and reached to 100% tumor incidence at 16th week. The first appearance of skin papillomas in groups topically treated with DHA (2.5 µmol) 30 min prior to each UVB exposure was recorded at 9th week and 100% of tumor incidence was observed at 18th week. The onset of papillomas and the development of 100% incidence in groups pretreated with DHA (10 µmol) were found to be at 8^{th} week and 20^{th} weeks, respectively after irradiation with UVB **(Fig. 4C)**. This data indicates that the UVB-induced skin tumor incidence was delayed by 2 or 4 weeks upon pretreatment with DHA. DHA also significantly decreased mouse skin tumor multiplicity in a dose-dependent manner compared to the group irradiated with UVB alone **(Fig. 4D)**. Moreover, repeated topical application of DHA prior to each UVB exposure reduced the cumulative tumor number of papillomas in mouse skin as compared with that of UVB radiation alone **(Fig. 4E)**.

DHA ameliorates histopathologic features of mouse skin papillomas

Exposure to UVB until 23 weeks completely disrupted the structure of epidermis and induced infiltration of inflammatory cells compared to normal mice. As evidenced by H&E staining (**Fig. 5, upper panel**). Repeated exposure to UVB also increased the 4-HNE-modified protein expression, which was attenuated by DHA pretreatment (**Fig. 5, middle panel**). Moreover, repeated application of DHA prior to UVB irradiation reduced the UVB-induced MDA levels in mouse skin papillomas (**Fig 5**, **bottom panel**).

DHA inhibits UVB-induced phosphorylation of STAT3 and expression of c-Myc in the skin papillomas

Since persistently activated STAT3 plays an important role in tumorigenesis through the upregulation of genes involved in anti-apoptosis, proliferation and angiogenesis, we examined the effect of DHA on STAT3 phosphorylation and c-Myc expression in UVB-induced mouse skin papillomas. DHA treatment significantly inhibited phosphorylation of STAT3 at tyrosine-705 residue in the skin papillomas compared with those from UVB alone-irradiated group **(Fig. 6A)**. Immunohistochemistry results also exhibited inhibition of UVB-induced STAT3 (Y705) phosphorylation by DHA **(Fig. 6B)**. Protein level of c-Myc which is a target gene product of STAT3, was decreased by DHA pretreatment **(Figure 6C)**.

DHA induces nuclear translocation of Nrf2 and enhances the expression of HO-1 and NQO-1 in HR-1 hairless mouse skin

Exposure to UVB is known to generate ROS and electrophiles that lead to oxidative and electrophilic stress, which can trigger an adaptive antioxidant response to protect cells from oxidative damage. Pretreatment of DHA increased the nuclear accumulation of Nrf2 in mouse skin **(Figure 6A)**. Since Nrf2 plays a key role in transcriptional activation of genes encoding various cytoprotective proteins, we examined the effect of DHA on the expression of two representative cytoprotective enzymes, HO-1 and NQO1. Topical application of DHA (2.5 or 10 μmol) increased the mRNA (**Fig. 6B**) and protein (**Fig. 6C**) expression of HO-1 and NQO1 in mouse skin. In addition, repeated application of DHA thrice a week for 23 weeks also induced the HO-1 expression in mouse skin (**Fig. 6D**).

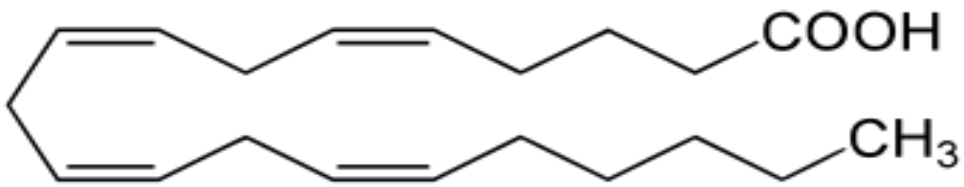

Figure 1. Chemical structure of of docosahexaenoic acid

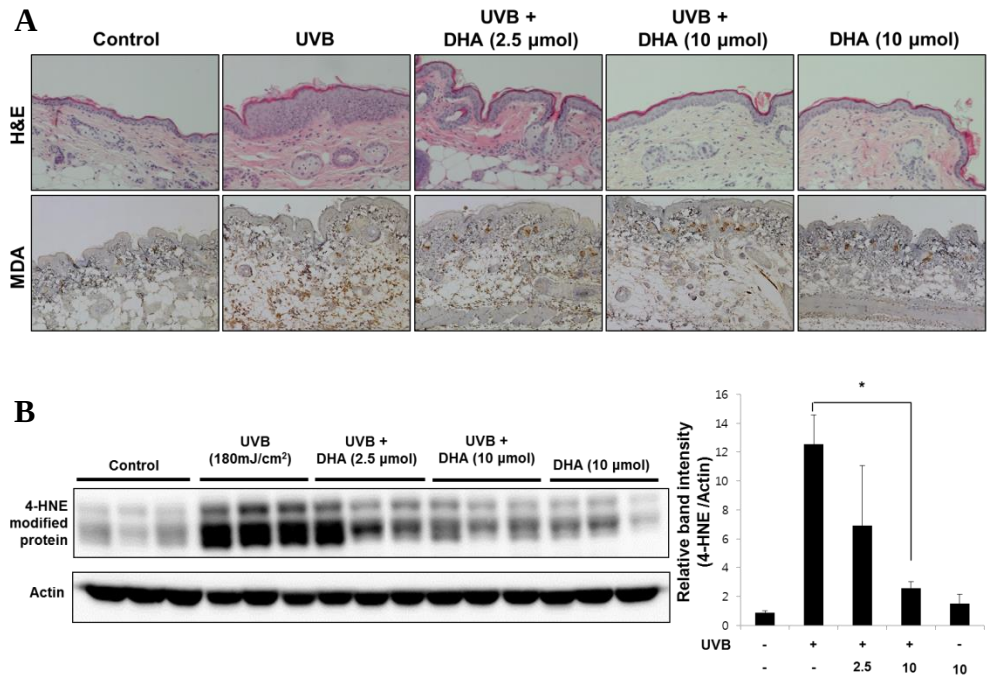

Figure 2. Inhibitory effects of DHA on UVB-induced epidermal hyperplasia and lipid peroxidation in HR-1 hairless mouse skin *in vivo*. Dorsal skin of female HR-1 hairless mice (*n* = 5 per treatment group) were treated topically with DHA (2.5 or 10 μmol) 30 min before UVB (180 mJ/cm^2) irradiation. Mice were sacrificed after 2 h of UVB irradiation. Control animals were treated with acetone alone and left unirradiated. (A) Irradiated skin tissue sections were subjected to H&E staining to examine inflammatory changes resulting in increased skin thickness. Formalin-fixed and paraffin-embedded tissues from UVB-irradiated mice were also immunostained for detecting the levels of MDA, and counterstained with

hematoxylin. Positive MDA staining yielded a brown-colored product. Magnifications X200. (B) Whole epidermal tissue lysates (30 μg protein) were separated by 10% SDS-PAGE and immunobloted for detecting 4-HNE-modified protein expression. Quantification of 4-HNE-modified proteins was normalized to that of actin followed by statistical analysis of relative band density. Data are expressed as means ± SE. *p <0.05.

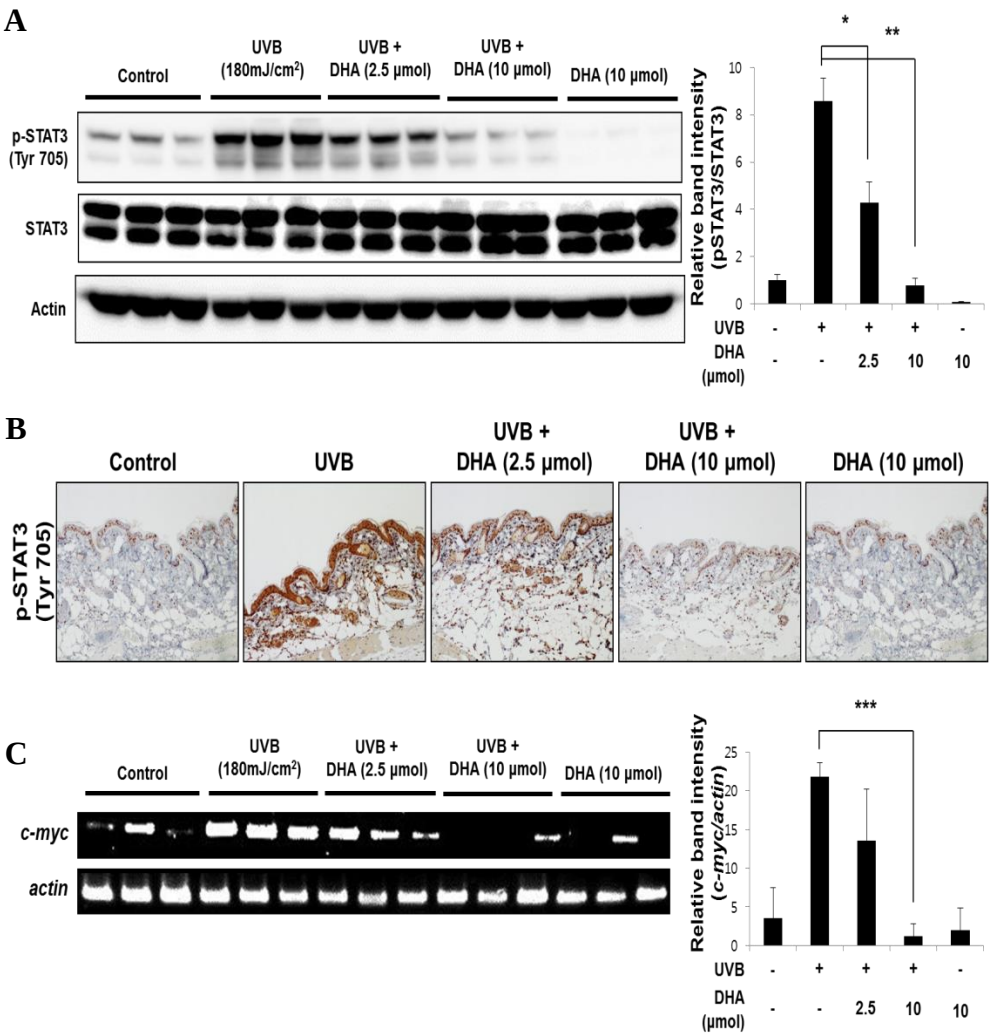

Figure 3. Effects of DHA on UVB-induced phosphorylation of STAT3 and expression of its target gene *c-myc* in the mouse skin. Animal treatment and other experimental conditions are same as described in the Figure 1 legend section. (A) Whole lysates (30 μg protein) from different treatment groups were separated by electrophoresis on 10% SDS-

polyacrylamide gel and immunoblotted to detect total and phosphorylated forms of STAT3 (Tyr^{705}). Quantification of p-STAT3 was normalized to that of STAT3 followed by statistical analysis of relative band density. (B) The sections of skin tissues were subjected to immunohistochemical analysis of phosphorylated of STAT3 at Tyr^{705}. Positive phosphorylation of STAT3 staining yielded a brown-colored product. Magnifications ×200. (C) Total RNA was isolated from differentially treated skin tissues using TRIzol® reagent according to the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to examine mRNA expression of *c-myc*. Quantification of *c-myc* was normalized to that of *actin* followed by statistical analysis of relative band density. Data are expressed as means ± SE. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

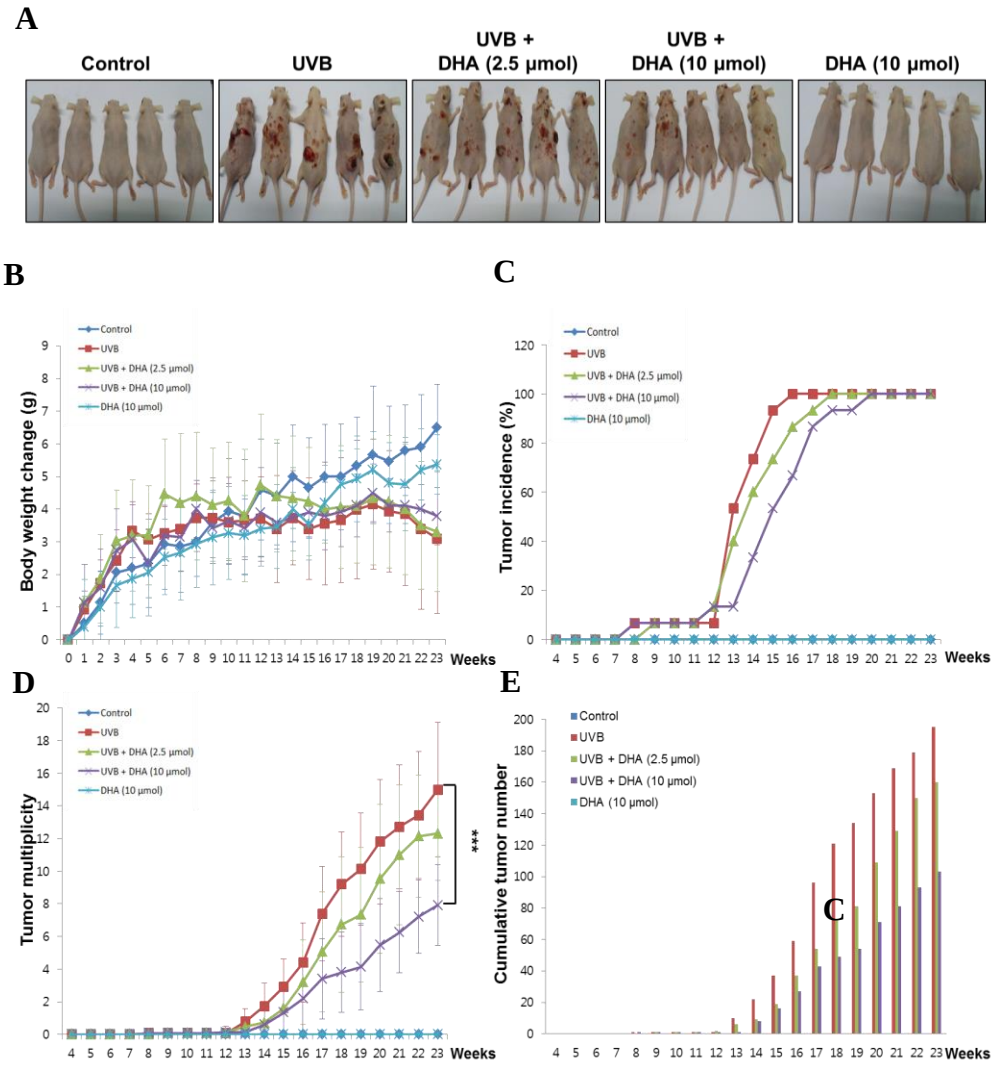

Figure 4. Effect of DHA on UVB-induced skin tumor development in HR-1 hairless mice. Female HR-1 hairless mice (*n* = 15 per treatment group) were topically treated on their backs with DHA (2.5 or 10 μmol) 30 min prior to UVB radiation (180 mJ/cm^2) three times a week until termination of

the experiment at 23rd week. (A) Representative phostographs of animals from different treatment groups, (B) The body weight change was measured on a weekly basis. Starting four week following UVB treatment, tumors of at least 1 mm diameter were monitored and counted every week till 23 weeks. The results were expressed as (C) the percentage of papilloma-bearing mice (incidence), (D) the average number of papillomas per mouse (multiplicity), and (E) the cumulative number of papillomas among tumor-bearing mice. Data are expressed as means ± SD. ***p <0.001.

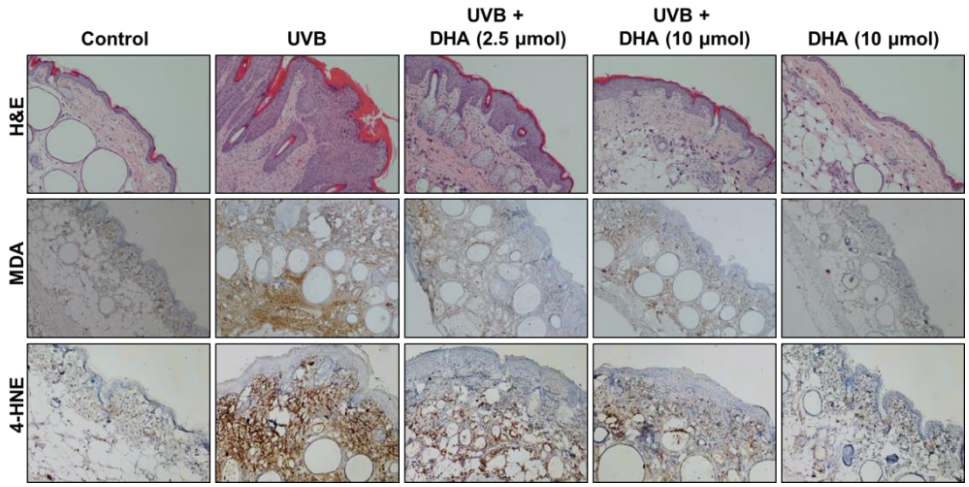

Figure 5. Effects of DHA on histopathologic features of mouse skin papillomas. H&E staining of papillomas showed hypertrophic squamous epithelium forming papillary fronds (upper panel). The respective sections were also immunostained for MDA (middle panel) and 4-HNE ((bottom panel), and counterstained with hematoxylin. Positive MDA and 4-HNE staining yielded a brown-colored product. Magnifications ×200 (H&E, 4-HNE), x100 (MDA).

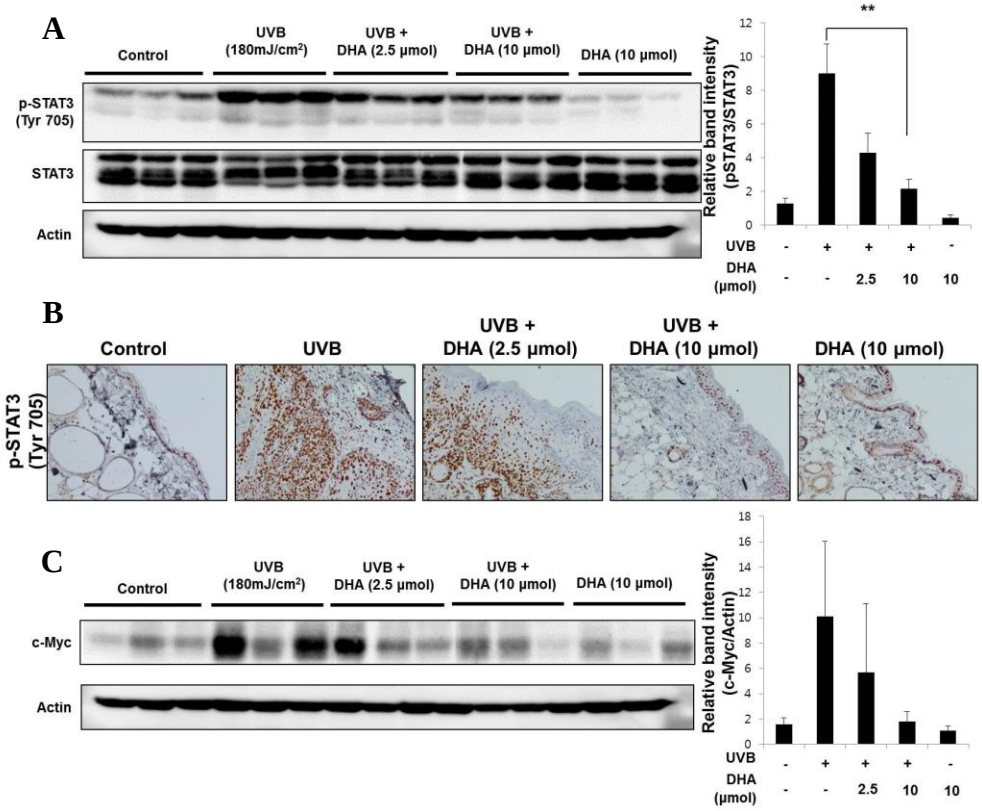

Figure 6. Effects of DHA on UVB-induced phosphorylation of STAT3 and expression of its target protein, c-Myc in the mouse skin papillomas. Animal treatment and other experimental conditions are described in the legend to Figure 3. (A) Whole surrounding tissue extracts (30 μg protein) were separated by electrophoresis on 10% SDS-polyacrylamide gel and immunoblotted to detect total and phosphorylated forms of STAT3 (Tyr^{705}). Quantification of pSTAT3 was normalized to that of STAT3 followed by statistical analysis of relative band density. Data are expressed as means ± SE. $^{**}p < 0.01$. (B) Formalin-fixed skin tissues pretreated with DHA and

exposed to UVB radiation were subjected to immunohistochemical analysis using a specific antibody to detect STAT3 phosphorylation at Tyr^{705}. Magnifications ×300. (C) Whole lysates (30 μg protein) were also analyzed for the protein expression of c-Myc by immunoblotting. Quantification of c-Myc immunoblot was normalized to that of actin followed by statistical analysis of relative band density.

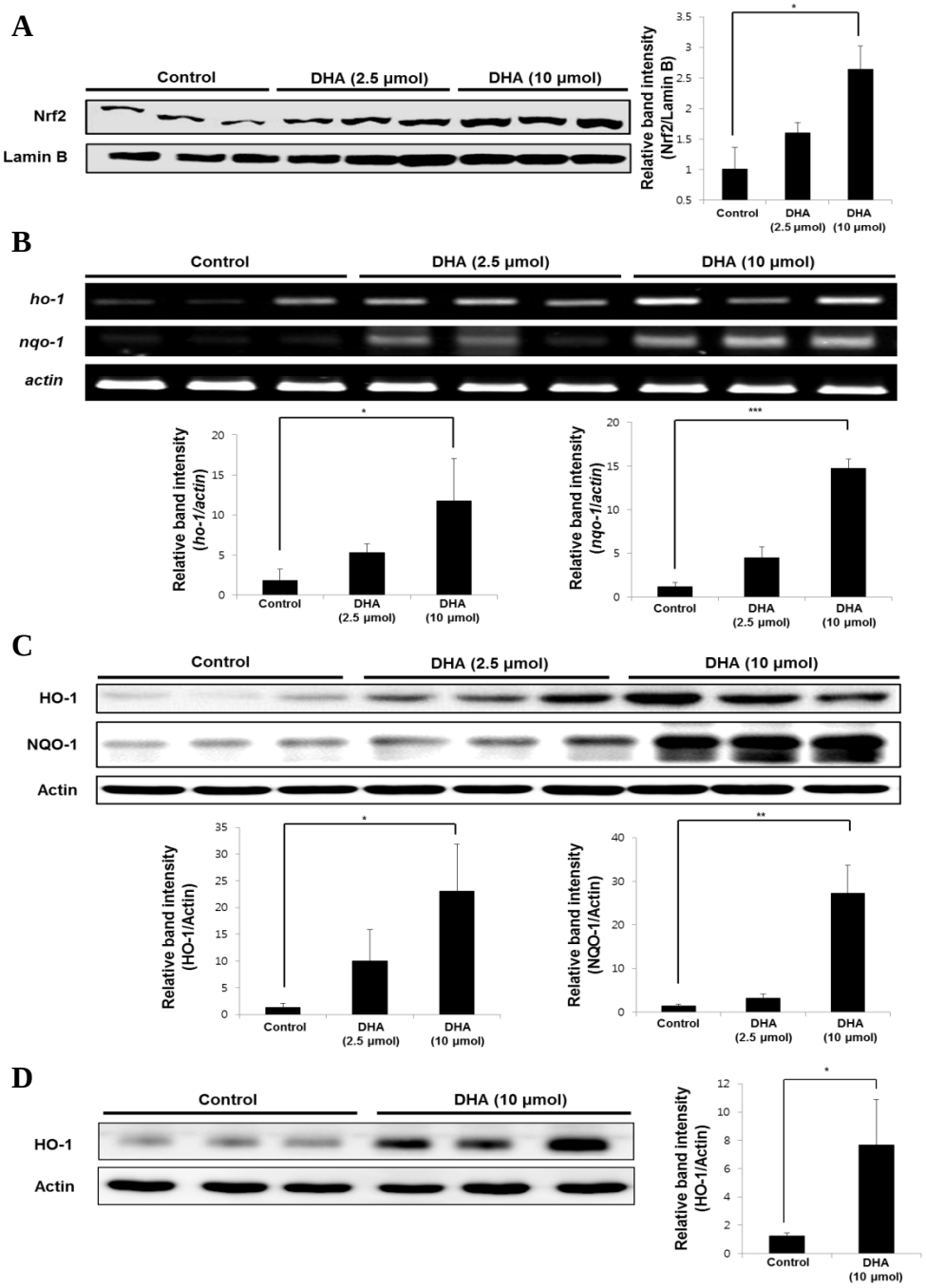

Figure 7. Effects of DHA on nuclear translocation of Nrf2 and the expression of HO-1 and NQO1 in HR-1 hairless mouse skin *in vivo*.

Dorsal skin of female HR-1 hairless mice (n = 5 per treatment group) were topically treated with DHA at the indicated doses. Control animals were treated with acetone in lieu of DHA. (A) After 2.5 h of treatment, the nuclear fraction was isolated and analyzed to determine the Nrf2 levels by western blotting. (B) Total RNA was isolated from skin tissues using TRIzol® reagent according to the manufacturer's protocol. RT-PCR analysis was performed to detect mRNA expression of *ho-1 and nqo-1*. Quantification of *ho-1 and nqo-1* was normalized to that of *actin* followed by statistical analysis of relative band density. (C) Epidermal tissue lysates (30 μg protein) were separated by electrophoresis on 10% SDS-polyacrylamide gel and immunoblotted to detect protein expression of HO-1. (D) After 23 weeks of treatment, whole tissue extracts (30 μg protein) were analyzed for the protein expression of HO-1 by immunoblotting. Quantification of HO-1 immunoblot was normalized to that of actin followed by statistical analysis of relative band density. Data are expressed as means ± SE. *p <0.05.

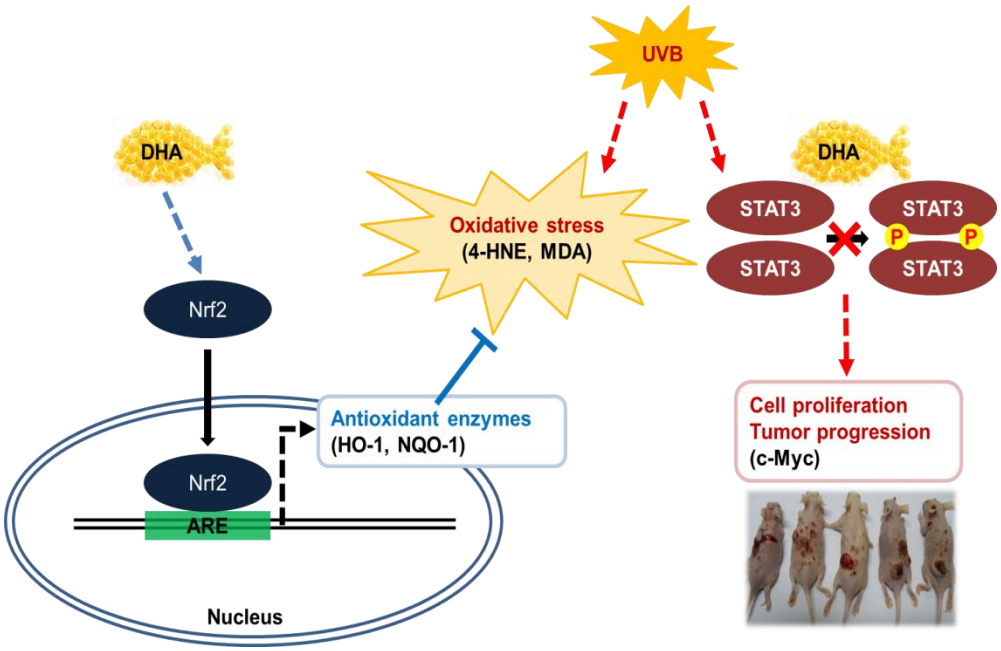

Figure 8. A proposed mechanism underlying the protective effects of DHA against UVB-induced inflammation and photocarcinogenesis in HR-1 hairless mouse skin

Discussion

The exposure to UVB radiation causes a variety of dermatologic disorders, such as inflammation, erythema, immunosuppression and cancer (Afaq et al. 2005, Halliday 2005, Matsumura and Ananthaswamy 2004). A wide variety of natural antioxidative and anti-inflammatory agents, in particular those present in our regular diet, have been shown to inhibit UVB-induced skin cancer (Mittal et al. 2003, Singh et al. 2006, Staniforth et al. 2006, Won et al. 2004). The ω-3 polyunsaturated fatty acids (PUFAs), present in fish oil and plant seed oil, possess a broad range of health beneficial properties such as antioxidant, anti-inflammatory and anti-cancer activities (Jho et al. 2004). DHA, a representative ω-3-PUFA, has been reported to inhibit UVB-induced oxidative and inflammatory responses in mouse skin (Rahman et al. 2011). Rahman *et al.* (2011) have demonstrated that topical application of DHA inhibited UVB-induced expression of NAD(P)H oxidase-4, an enzyme involved in the generation of ROS, and that of a pro-inflammatory enzyme cyclooxygenase-2 (COX-2) in hairless mouse skin. In line with study, we found that topical application of DHA attenuated epidermal hyperplasia and reduced the accumulation of lipid peroxidation products, such as MDA and 4-HNE in UVB-irradiated mouse skin. These findings are in good agreement

with the previously published antioxidative effects of the compound (Chen et al. 2013, Sunada et al. 2006).

Our study revealed for the first time that DHA treatment inhibited the incidence and the multiplicity of UVB radiation-induced mouse skin papillomagenesis. The inhibitory effects of DHA on the UVB-induced increase in MDA and 4-HNE levels in skin papillomas indicate that DHA suppress papilloma formation by virtue of its antioxidant effects in UVB-irradiated mouse skin.

Ahsan et al. (2005) have demonstrated that exposure to UVB radiation phosphorylate STAT3 at tyrosine 705 residue in SKH1 hairless mouse skin. Moreover, STAT3 activation promotes keratinocyte survival and proliferation in response to UVB radiation (Sano et al. 2005). In addition, Kim et al. (2009) reported that constitutive activation of STAT3 enhances UVB-induced skin carcinogenesis. Thus, inhibition of STAT3 signaling is considered as a pragmatic approach for the prevention of UVB-induced skin carcinogenesis. Our finding that DHA treatment diminished STAT3 phosphorylation at tyrosine 705 residue as well as the mRNA expression of its target gene product, *c-myc*, in acute or chronic UVB-irradiated mouse skin or papillomas, respectively, suggest that DHA exerts its skin cancer chemopreventive effects, at least in part, by blocking STAT3 activation.

The dimerization and subsequent transcriptional activation of STAT3

requires its phosphorylation at tyrosine-705 and serine-727 residues. While tyrosine phosphorylation of STAT3 is mediated largely by upstream Janus-activated kinases (Jak) (Aaronson and Horvath 2002), its serine phosphorylation is regulated by serine/threonine kinases (Turkson et al 1999, Zhang et al 2001). Among the serine/threonine kinase series of mitogen-activated protein (MAP) kinases, c-Jun-N-terminal kinase (JNK), but not extracellular kinase (ERK) or p38 MAP kinase, has been reported to regulate STAT3 serine phosphorylation in UVA-stimulated mouse epidermal (JB6) cells (Zhang et al 2001). Rahman et al. (Rahman et al 2011) reported that DHA inhibited UVB-induced phosphorylation of ERK and p38 MAP kinase in mouse skin. Thus, the effect of DHA on UVB-induced STAT3 phosphorylation at serine 727 residue merits further investigation.

Although UVB-induced ROS are involved in skin photodamage, body protects against oxidative stress through activation of a series of antioxidant and detoxification enzymes, collectively known as cytoprotective proteins. HO-1 and NQO1 are two representative cytoprotective proteins, which are transcriptionally regulated by a redox sensitive transcription factor Nrf2 (Surh et al 2008). Was et al. (Was et al 2011) demonstrated that mice expressing wild type HO-1 exhibited relatively reduced multiplicity of skin papillomas as compared to HO-1 knockout animals at the early stage of skin carcinogenesis. Long et al. (Long et al 2000, Long et al 2001) reported that

NQO1-null mice are more sensitive to chemically induced skin tumorigenesis as compared to wild type mice. Likewise, Nrf2 knockout mice are more susceptible to develop chemically induce skin tumors (Xu et al 2006). Since the induction of HO-1 and NQO1 elicit antioxidative and anti-inflammatory effects (Surh et al 2008), our findings that DHA increases the nuclear accumulation of Nrf2 and induces the expression of HO-1 and NQO1 in mouse skin may be attributed to its inhibitory effects on UVB-induced skin carcinogenesis.

In conclusion, the present study demonstrates that topical application of DHA protected against UVB-induced papillomagenesis by suppressing oxidative and inflammatory damages in mouse skin through the inhibition of lipid perdoxidation and downregulation of STAT3 activation **(Figure 8)**.

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국문초록

UVB 에 의해 유도된 마우스 피부발암과정에서 Docosahexaenoic Acid 의 암예방 효과

암의 발생과 진행에 있어서 지속적인 염증반응은 매우 중요한 연관성이 있는 것으로 알려져 있다. 자외선 종류 중 하나인 Ultraviolet B (UVB)는 피부에 노출 시 염증반응을 유도하는 것으로 보고되고 있다.

Docosahexaenoic acid (DHA)은 오메가-3 지방산 중의 대표적인 물질로 등푸른 생선 및 계란 등에 많이 포함되어 있다. 또한 DHA 는 일반적으로 뇌의 발달, 항 산화, 항 노화, 항 염증, 항암작용 등이 있는 것으로 알려져 있다.

본 연구자는 마우스 피부 암화과정에 대한 DHA 의 예방 효과를 알아보기 위해 UVB 로 유도된 마우스 피부염 모델과 피부암 모델을 이용하여 검토하였다.

암컷 HR-1 마우스에 DHA 를 Acetone 에 희석하여 2.5 μmol 와 10 μmol 두 가지 농도로 마우스 등에 도포한 후, 30 분 경과 후

UVB (180 mJ/cm^2) 를 쬐도록 하였다. 피부염의 모델에서 hematoxylin and eosin (H&E)로 염색하여 피부 조직을 분석한 결과 DHA 가 UVB 에 의해 유도된 염증적 조직손상을 감소시키는 것을 관찰하였다. 또한 대표적인 염증관련 lipidperoxidation marker 인 4-HNE 와 MDA 가 DHA 에 의해 그 수준이 감소됨을 확인 하였다. DHA 의 도포가 마우스 피부에서 UVB 에 의해 signal transducer and activator of transcription 3 (STAT3)의 활성화 및 이들 전사인자들의 조절을 받는 표적 단백질들의 발현을 경감시키는 것 또한 확인하였다.

피부암의 모델에서 DHA 의 예방효과를 관찰하기 위하여 본 연구자는 다음과 같이 실험하였다. 일주일에 3 번, 23 주 동안 암컷 HR-1 마우스에 DHA 를 2.5 μmol 와 10 μmol 두 가지 농도로 마우스 등에 도포한 후, 30 분 경과 후 UVB (180 mJ/cm^2) 를 쬐도록 하였다. UVB 는 HR-1 mouse 에서 유의적으로 papilloma 형성을 촉진하였으며 이는 DHA 에 의해 그 수가 감소하였다. 또한 H&E 염색을 통하여 많은 염증관련 세포들이 피부에 많이 침윤되었음을 확인 하였으며 피부염 모델에서와

마찬가지로 4-HNE, MDA 의 수준이 DHA 에 의해 감소되었고 STAT3 의 활성화도 경감되었다.

별도의 실험에서 DHA 단독 도포 시 항 산화 효소의 발현에 관여하는 nuclear factor-erythroid related factor-2 (Nrf2)의 수준이 증가됨을 확인 하였으며, Nrf2 전사인자가 유도하는 hemeoxygenase-1 (HO-1) 와 NAD(P)H quinoneoxidoreductase-1 (NQO1) 수준 또한 발현이 증가되었다.

이와 같은 결과들은 DHA 가 UVB 에 의해 유도된 STAT3 의 활성화를 경감시키고, 항 산화 효소의 발현을 유도하여 UVB 가 유도한 산화적 스트레스를 감소시켜 마우스의 피부암에 예방효과를 나타냄을 시사한다.

주요어 : UVB, 피부 암화, STAT3, DHA, 항산화 효소

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藥學碩士學位論文

Protective Effects of Docosahexaenoic Acid against UVB-induced Mouse Skin Carcinogenesis

UVB 에 의해 유도된 마우스 피부발암과정에서 Docosahexaenoic Acid 의 암예방 효과

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朴珍

Abstract

Protective Effects of Docosahexaenoic Acid against UVB-induced Mouse Skin Carcinogenesis

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Exposure to solar radiation, especially ultraviolet B (UVB), is the most prevalent environmental carcinogen that increases the risk of skin cancer. Oxidative stress and persistent inflammation are the key pathologic events in UVB-induced skin photocarcinogenesis. Signal transducer and activator of transcription-3 (STAT3) 3, one of the major redox-sensitive transcription factors, plays an essential role in the pathogenesis of inflammation and subsequent development of skin cancer. Omega-3 polyunsaturated fatty acids

(ω-3 PUFAs), which occur at high levels in some fish oils, are known to possess radical scavenging activity and increase host immunoresponsiveness. The present study was aimed at evaluating the anti-inflammatory and anti-tumor promoting activity of docosahexaenoic acid (DHA), a prototypic ω-3 PUFA, in mouse skin irradiated with UVB. Topical application of DHA (2.5 and 10 μmol) prior to UVB irradiation (180 mJ/cm^2) diminished epidermal hyperplasia and formation of 4-hydroxynonenal modified protein and malondialdehyde, the biochemical hallmarks of lipid peroxidation, in HR-1 hairless mouse skin. Pretreatment with DHA inhibited UVB-induced phosphorylation of STAT3 (Tyr^{705}) and expression of its target gene, *c-myc* in the mouse skin. Moreover, repeated DHA pretreatment until the termination of the experiment at the 23rd week reduced the incidence and the multiplicity of papillomas and squamous cell carcinomas in UVB-induced mouse skin. DHA down-regulates STAT3 activation and c-Myc expression in papillomas as compared to ultraviolet radiation alone. Furthermore, topically applied DHA induced the expression of antioxidant enzymes, heme oxygenase-1 and NAD(P)H:quinone oxidoreductase 1 in the mouse skin. Taken together, DHA protected against UVB-induced mouse skin tumor development by blocking STAT3 activation and enhancing inducing antioxidant enzyme expression, suggesting that this ω-3 PUFA has potential for use to ameliorate UVB-induced skin tissue damage and related abnormal disorders including

photocarcinogenesis.

Keywords: UVB, Skin carcinogenesis, STAT3, DHA, Antioxidant enzymes

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Introduction

Ultraviolet (UV) radiation is an important environmental factor responsible for the pathogenesis of skin aging and photocarcinogenesis. According to the *World Cancer Report*, skin cancer constitutes approximately 30% of all newly diagnosed cancers in the world. Exposure to UVB radiation is the major cause of 90% of skin cancers. Irradiation with UVB induces oxidative stress and inflammatory tissue damage, thereby causing squamous and basal cell carcinomas (Afaq et al. 2005, Halliday 2005). UVB exerts its detrimental effect mainly through direct DNA damage and the generation of reactive oxygen species (ROS), which cause oxidative or covalent modification of cellular macromolecules, such as lipids, proteins and nucleic acids. For example, the UVB irradiation increases the accumulation of lipid peroxidation products, such as 4-hydroxynonenal (4HNE) and malondialdehyde (MDA) (Oberley et al. 2004, Petersen and Doorn 2004, Tyrrell 1995). Moreover, UVB-induced generation of ROS amplify intracellular signaling pathways comprising various kinases and transcription factors (Bachelor et al. 2005, Bode and Dong 2003, Dickinson et al. 2011). One of the UVB-responsive transcription factors is signal transducer and activator of transcription-3 (STAT3), which promotes the transcriptional

upregulation of genes involved in cell proliferation and tissue inflammation (Kundu and Surh 2012). The activation of STAT3 is dependent on the phosphorylation of tyrosine-705 (Y705) residue and serine-727 (S727) residue. Upon phosphorylation of these serine and tyrosine residues, STAT3 is dimmerized and translocated into the nucleus, where its binds to the promoter regions of genes encoding cell survival proteins, such as cyclins, c-Myc, survivin, Bcl-2, Bcl-xl (Aaronson and Horvath 2002, Turkson et al. 1999, Zhang et al. 2001). Aberrant activation of STAT3 has been implicated in UVB-induced skin carcinogenesis (Kim et al. 2009). Thus, the normalization of inappropriately amplified STAT3 signaling cascade might be a rational approach for chemoprevention of skin carcinogenesis.

Since UVB-induced ROS trigger skin photo-inflammation and photocarcinogenesis, the mitigation of oxidative stress can protect cellular macromolecules from oxidative damage or covalent modification. A battery of endogenous antioxidants maintains the cellular redox balance. These include a series of antioxidants and detoxification enzymes, collectively called cytoprotective proteins, are involved in the hermetic control of cellular redox status (Surh et al. 2008). For instance, hemeoxygenase-1 (HO-1) and NAD(P)H quinoneoxidoreductase-1 (NQO1) have been reported to protect skin from oxidative and inflammatory tissue injury and carcinogenesis. A recent study demonstrated that mice harboring wild type HO-1 are less

susceptible to chemical carcinogen-induced skin inflammation and tumor formation in the early stage of papillomagenesis as compared to HO-1 knockout mice (Was et al. 2011). Likewise, the NQO1-null mice are more susceptible to chemically induced skin tumor development (Long et al. 2000, Long et al. 2001). Thus, the fortification of the expression and/or activity of HO-1 and NQO1 is a practical approach to prevent skin carcinogenesis. The promoter regions of genes encoding HO-1 and NQO1 harbors *cis*-acting regulatory sequences, known as antioxidant response element (ARE) or electrophile response element (EpRE) (Surh et al. 2008). Mild oxidative and/or electrophilic stress activates a redox-sensitive transcription factor nuclear factor-erythroid related factor-2 (Nrf2), which by interacting with the ARE sequences upregulate the expression of cytoprotctive proteins, such as HO-1 and NQO1 (Surh et al. 2008). Thus, one of the potential strategies to prevent skin cancer is to enhance the Nrf2-mediated induction of cytoprotective proteins.

A wide variety of dietary antioxidant and anti-inflammatory substances have been reported to prevent skin cancer (Gupta and Mukhtar 2001). A class of promising dietary chemopreventive substances is ω-3 polyunsaturated fatty acids (PUFAs) present in fish oil and plant-based diets including flaxseed oil. Docosahexaenoic acid (DHA) (**Fig. 1**), a representative ω-3 PUFAs, has been extensively investigated for health beneficial effects, such as antioxidative,

anti-inflammatory, neuroprotective and chemopreventive activities (Jho et al. 2004, Rahman et al. 2011). The effects of DHA on UVB-induced skin photocarcinogenesis and its underlying mechanisms have not been investigated yet. Here, we report that topical application of DHA inhibits UVB-induced skin papillomagenesis, partly, by blocking STAT3 signaling and inducing Nrf2-mediated expression of HO-1 and NQO1.

Materials and Methods

Materials

DHA (purity > 98%) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Primary antibodies for pSTAT3 and STAT3 were procured from Cell Signaling Technology (Beverly, MA, USA). Antibodies for c-Myc, Nrf2 and HO-1 were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies for Lamin-B was purchased from BD Biosciences (San Jose, CA, USA). Antibodies of 4-HNE-modified proteins and MDA were obtained from Japan Institute for the Control of Aging (Shizuoka, Japan). Anti-actin antibody was obtained from Sigma Chemical Company (St. Louis, MO, USA). Antibody against NQO1 was obtained from Abcam (Cambridge, UK). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were obtained from Zymed Laboratories Inc. (San Francisco, CA, USA). Enhanced chemiluminescent (ECL) detection kit and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals used were in the purest form available commercially.

Animal treatment

Female HR-1 hairless mice (6-7 weeks age) were supplied from Sankyo Laboservice Corporation, Inc. (SLC, Tokyo, Japan). Animals were housed in climate-controlled quarters (24 ± 1^{0}C at 50% humidity) with a 12-h light/12-h dark cycle. DHA (2.5 and 10 µmol) was dissolved in 200 µl of acetone and applied topically to the dorsal skin 40 min before exposure to UVB (180 mJ/cm^2) radiation.

Source of UVB radiation

The UVB radiation was a 5 × 8 Watt tube, which emits an energy spectrum with high fluency in the UVB region (with a peak at 312 nm). A Biolink BLX-312 UV crosslinker (Vilbert Lourmat, Marne-la-Valée, France) was used in the present study to irradiate mouse skin.

Western blot analysis

Dorsal skin of HR-1 hairless mice were treated with DHA (2.5 or 10 µmol/mouse) 40 min before exposure to UVB (180 mJ/cm^2) and sacrificed by cervical dislocation 2.5 h later. Control animals were treated with vehicle only. For the preparation of mouse epidermal protein extract, fat and dermis were removed from the harvested skin samples kept on ice and the fat-free epidermis was immediately placed in liquid nitrogen and pulverized in mortar. The pulverized skin was homogenized on ice for 20 s with a polytron

tissue homogenizer and lysed in 1 mL ice-cold lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl pH 7.4, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4 and protease inhibitor cocktail tablet). Lysates were centrifuged at 14,800 *g* for 15 min. The supernatant was collected and total protein concentration was quantified by using the bicinchoninic acid (BCA) protein assay kit. Cell lysates (30 µg protein) were boiled in sodium dodecyl sulfate (SDS) sample loading buffer for 5 min before electrophoresis on 10-12% SDS-polyacrylamide gel. After transfer to polyvinylidene difluoride (PVDF) membrane, the blots were blocked with 5% fat-free dry milk-PBST (Phosphate-buffer saline containing 0.1% Tween 20) or 1% bovine serum albumin (BSA) in TBST (Tris-buffer saline containing 0.1% Tween 20) for 1 h at room temperature and then washed with PBST or TBST buffer. The membranes were incubated for 2 h at room temperature with 1:1000 dilutions of primary antibodies for actin and for 12 h at 4 ^{0}C with 1:1000 dilutions of primary antibodies for 4-HNE modified protein, c-Myc, Nrf2, NQO-1, Lamin B, STAT3 and phosho-STAT3. Blots were washed three times with PBST or TBST at 5 min intervals followed by incubation with 1:5000 dilution of respective horseradish peroxidase conjugated secondary antibodies (rabbit, goat or mouse) in 3% fat-free dry milk-TBST for 1 h at room temperature. The blots were rinsed again three times with PBST or TBST. The immunoblots were visualized with an ECL detection kit

according to the manufacturer's instructions.

Histological analysis

Sections of harvested mouse skin were washed with phosphate-buffered saline (PBS) and fixed with 10% buffered formalin and embedded in paraffin. Each section (4 μm) was stained with hematoxylin and eosin (H&E). The H&E stained sections were examined under light microscope to detect the presence of lesions.

Immunohistochemical analysis

Mouse skin irradiated with UVB in presence or absence of DHA were subjected to immunohistochemical analysis for detecting the expression of 4-HNE modified protein, MDA and phospho-STAT3. Four-micrometer sections of 10% formalin-fixed, paraffin-embedded tissues were cut on salinized glass slides and deparaffinized three times with xylene, and rehydrated through graded alcohol bath. The deparaffinized sections were heated with microwave and boiled twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish non-specific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. For the detection of respective protein expression, slides were incubated with affinity purified rabbit polyclonal anti-4-HNE, anti-

MDA or anti-pSTAT3 (Y705) (1:50) at room temperature for 40 min in Tris-buffered saline containing 0.05% Tween 20 and then developed using anti-rabbit HRP EnVision^{TM} System (Dako, Glostrup, Denmark). The peroxidase binding sites were detected by staining with 3,3'-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark). Finally, counterstaining was performed using Mayer's hematoxylin.

Reverse transcriptase polymerase chain reaction

Total RNA was isolated from skin tissue by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. To generate the cDNA from RNA, 1 μg of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase (Promega) for 50 min at 42℃ and again for 15 min at 72℃. One microliter of cDNA was amplified in sequential reactions by using Maxime PCR PreMix Kit (iNtRON Biotechnology). For detection of HO-1 mRNA, 20 cycles of 94℃ for 30 seconds, 53℃ for 35 seconds, and 72℃ for 30 seconds were conducted; for quantitation of actin mRNA, 20 cycle of 94℃ for 30 seconds, 59℃ for 35 seconds, and 72℃ for 30 seconds were conducted. These PCR cycles were followed by a final extension for 7 minutes at 72℃. The primers used for each RT-PCR reactions are as follows: *HO-1*, 5′-TAC ACA TCC AAG CCG AGA AT-3′ and 5′-GTT CCT CTG

TCA GCA TCA CC-3′; *Nrf2,* 5′-TGC CCC TCA TCA GGC CCA GT-3′ and 5′-GCT CGG CTG GGA CTC GTG TT-3′; *NQO-1*, 5′-GAG GAC CTC CTT CAA CTA TG-3′ and 5′-CCTTTG TCA TAC ATG GCA GC-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). Amplification products were analyzed by 2.0% agarose gel electrophoresis, followed by staining with ethidium bromide, and then photographed under UV light.

Preparation of cytosolic and nuclear extracts

The cytosolic and nuclear extract from mouse skin was prepared as described previously. In brief, scraped dorsal skin of mice was homogenized in 800 μl of hypotonic buffer A [10 mM HEPES, pH 7.8; 10 mM KCl; 2 mM $MgCl_{2;}$ 1 mM DTT; 0.1 mM EDTA; 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. To the homogenates was added 80 μl of 10% Nonidet P-40 (NP-40) solution, and the mixture was then centrifuged for 2 min at 14000 *g*. The supernatant was collected as cytosolic fraction. The precipitated nuclei were washed once with 500 μL of buffer A plus 40 μl of 10% NP-40, centrifuged, resuspended in 200 μl of buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 20% glycerol] and centrifuged for 5 min at 14,800*g*. The supernatant containing nuclear

proteins was collected and stored at -70°C after determination of protein concentrations.

Statistical analysis

Values were expressed as the mean ± SEM of at least three independent experiments. Statistical significance was determined by Student's *t* test and a *p*-value of less than 0.05 was considered to be statistically significant.

Results

DHA attenuates UVB-induced lipid peroxidation and skin hyperplasia in female HR-1 mouse skin in vivo

UVB irradiation is known to produce ROIs, which leads to lipid peroxidation (Halliday 2005). Multiple lines of evidence suggest that UVB-induced generation of ROS, such as superoxide anion, hydroxyl radical and hydrogen peroxide, is responsible for skin hyperplasia (Tyrrell 1995). The 4-HNE and MDA are representative lipid peroxidation products (Oberley et al. 2004, Petersen and Doorn 2004). Immunohistochemical analysis of mouse skin irradiated with UVB in presence or absence of DHA (2.5 or 10 μmol) revealed that pretreatment with DHA attenuated UVB-induced skin hyperplasia (**Fig. 2A**, upper panel) and the levels of MDA (**Fig. 2A,** lower panel). DHA pretreatment also inhibited UVB-induced accumulation of 4-HNE-modified proteins in mouse epidermis (**Fig. 2B**).

DHA inhibits UVB-induced activation of STAT3 and expression of its target gene, c-myc in hairless mouse skin

Aberrant activation of STAT3 signaling has been implicated in skin carcinogenesis (Kim et al. 2009). It has been reported that exposure to UVB

radiation activates STAT3 via phosphorylation at tyrosine-705 residue in SKH1 hairless mouse skin (Ahsan et al. 2005). We, therefore, examined the effect of DHA on UVB-induced activation of STAT3 and the expression of its target gene c-Myc. As show in **Fig. 3A**, irradiation of HR-1 hairless mouse skin with UVB (180 mJ/cm^2) increased the phosphorylation of STAT3 at tyrosine-705 residue, which was blunted by pretreatment with DHA (2.5 or 10 μmol) in a dose-dependent manner. The inhibitory effect of DHA on UVB-induced STAT3 (Y705) phosphorylation was verified by immunohistochemical analysis. As shown in **Fig. 3B**, UVB irradiation markedly increased the expression of pSTAT3(Y705) appeared as brown color staining. Pretreatment with DHA attenuated UVB-induced expression of pSTAT3(Y705), while treatment with DHA alone maintained the pSTAT3(Y705) level comparable to that of control. Moreover, UVB irradiation elevated the mRNA expression of a STAT3 target gene, *c-Myc*, which was significantly diminished by pretreatment with DHA (**Fig. 3C**).

DHA inhibits UVB-induced papillomagenesis in HR-1 hairless mouse skin

Since DHA attenuated UVB-induced lipid peroxidation, skin hyperplasia and STAT3 activation, we examined the effect of repeated application of DHA on papillomagenesis in a UVB-induced skin carcinogenesis model. At the termination of experiment after 23 weeks, representative photographs of

mice from indicated treatment groups were taken. **Fig. 4A** shows that UVB irradiation alone induced skin papilloma formation, which was diminished by DHA pretreatment. We monitored the body weight of each mouse every week until termination of the experiment. The body weight change of UVB-treated mice was decreased compared with control group. However, administration of DHA prevented UVB-induced body weight loss **(Fig. 4B)**. Animals irradiated with UVB alone developed skin papillomas at 8th week and reached to 100% tumor incidence at 16th week. The first appearance of skin papillomas in groups topically treated with DHA (2.5 µmol) 30 min prior to each UVB exposure was recorded at 9th week and 100% of tumor incidence was observed at 18th week. The onset of papillomas and the development of 100% incidence in groups pretreated with DHA (10 µmol) were found to be at 8^{th} week and 20^{th} weeks, respectively after irradiation with UVB **(Fig. 4C)**. This data indicates that the UVB-induced skin tumor incidence was delayed by 2 or 4 weeks upon pretreatment with DHA. DHA also significantly decreased mouse skin tumor multiplicity in a dose-dependent manner compared to the group irradiated with UVB alone **(Fig. 4D)**. Moreover, repeated topical application of DHA prior to each UVB exposure reduced the cumulative tumor number of papillomas in mouse skin as compared with that of UVB radiation alone **(Fig. 4E)**.

DHA ameliorates histopathologic features of mouse skin papillomas

Exposure to UVB until 23 weeks completely disrupted the structure of epidermis and induced infiltration of inflammatory cells compared to normal mice. As evidenced by H&E staining (**Fig. 5, upper panel**). Repeated exposure to UVB also increased the 4-HNE-modified protein expression, which was attenuated by DHA pretreatment (**Fig. 5, middle panel**). Moreover, repeated application of DHA prior to UVB irradiation reduced the UVB-induced MDA levels in mouse skin papillomas (**Fig 5**, **bottom panel**).

DHA inhibits UVB-induced phosphorylation of STAT3 and expression of c-Myc in the skin papillomas

Since persistently activated STAT3 plays an important role in tumorigenesis through the upregulation of genes involved in anti-apoptosis, proliferation and angiogenesis, we examined the effect of DHA on STAT3 phosphorylation and c-Myc expression in UVB-induced mouse skin papillomas. DHA treatment significantly inhibited phosphorylation of STAT3 at tyrosine-705 residue in the skin papillomas compared with those from UVB alone-irradiated group **(Fig. 6A)**. Immunohistochemistry results also exhibited inhibition of UVB-induced STAT3 (Y705) phosphorylation by DHA **(Fig. 6B)**. Protein level of c-Myc which is a target gene product of STAT3, was decreased by DHA pretreatment **(Figure 6C)**.

DHA induces nuclear translocation of Nrf2 and enhances the expression of HO-1 and NQO-1 in HR-1 hairless mouse skin

Exposure to UVB is known to generate ROS and electrophiles that lead to oxidative and electrophilic stress, which can trigger an adaptive antioxidant response to protect cells from oxidative damage. Pretreatment of DHA increased the nuclear accumulation of Nrf2 in mouse skin **(Figure 6A)**. Since Nrf2 plays a key role in transcriptional activation of genes encoding various cytoprotective proteins, we examined the effect of DHA on the expression of two representative cytoprotective enzymes, HO-1 and NQO1. Topical application of DHA (2.5 or 10 μmol) increased the mRNA (**Fig. 6B**) and protein (**Fig. 6C**) expression of HO-1 and NQO1 in mouse skin. In addition, repeated application of DHA thrice a week for 23 weeks also induced the HO-1 expression in mouse skin (**Fig. 6D**).

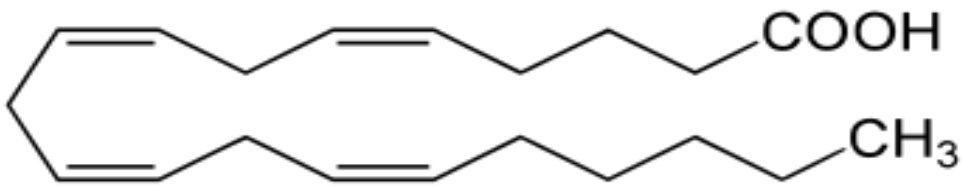

Figure 1. Chemical structure of of docosahexaenoic acid

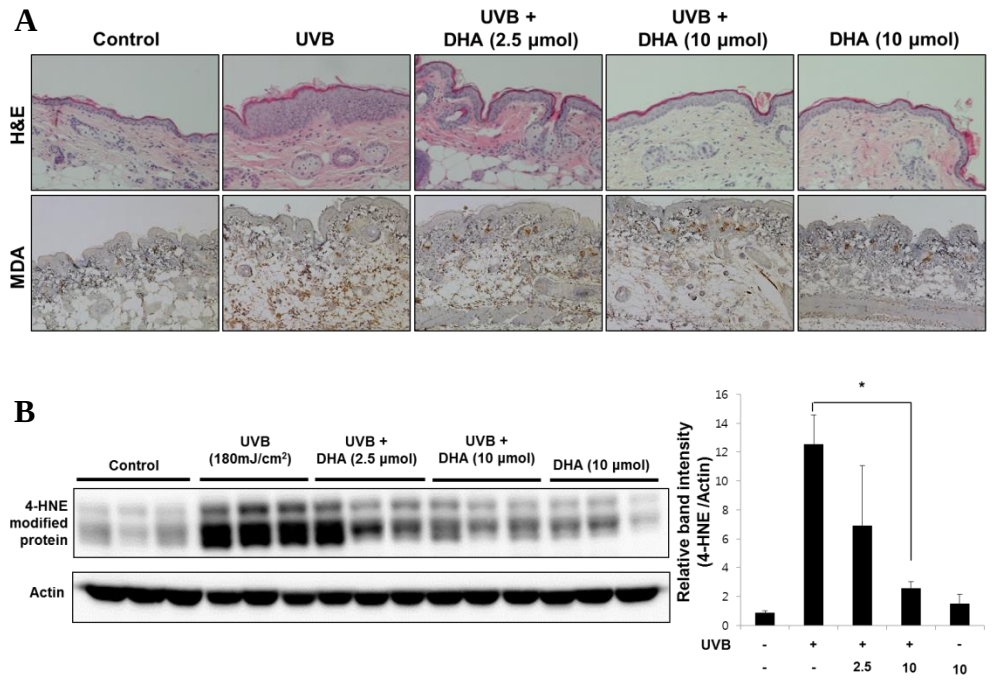

Figure 2. Inhibitory effects of DHA on UVB-induced epidermal hyperplasia and lipid peroxidation in HR-1 hairless mouse skin *in vivo*. Dorsal skin of female HR-1 hairless mice (*n* = 5 per treatment group) were treated topically with DHA (2.5 or 10 μmol) 30 min before UVB (180 mJ/cm^2) irradiation. Mice were sacrificed after 2 h of UVB irradiation. Control animals were treated with acetone alone and left unirradiated. (A) Irradiated skin tissue sections were subjected to H&E staining to examine inflammatory changes resulting in increased skin thickness. Formalin-fixed and paraffin-embedded tissues from UVB-irradiated mice were also immunostained for detecting the levels of MDA, and counterstained with

hematoxylin. Positive MDA staining yielded a brown-colored product. Magnifications X200. (B) Whole epidermal tissue lysates (30 μg protein) were separated by 10% SDS-PAGE and immunobloted for detecting 4-HNE-modified protein expression. Quantification of 4-HNE-modified proteins was normalized to that of actin followed by statistical analysis of relative band density. Data are expressed as means ± SE. *p <0.05.

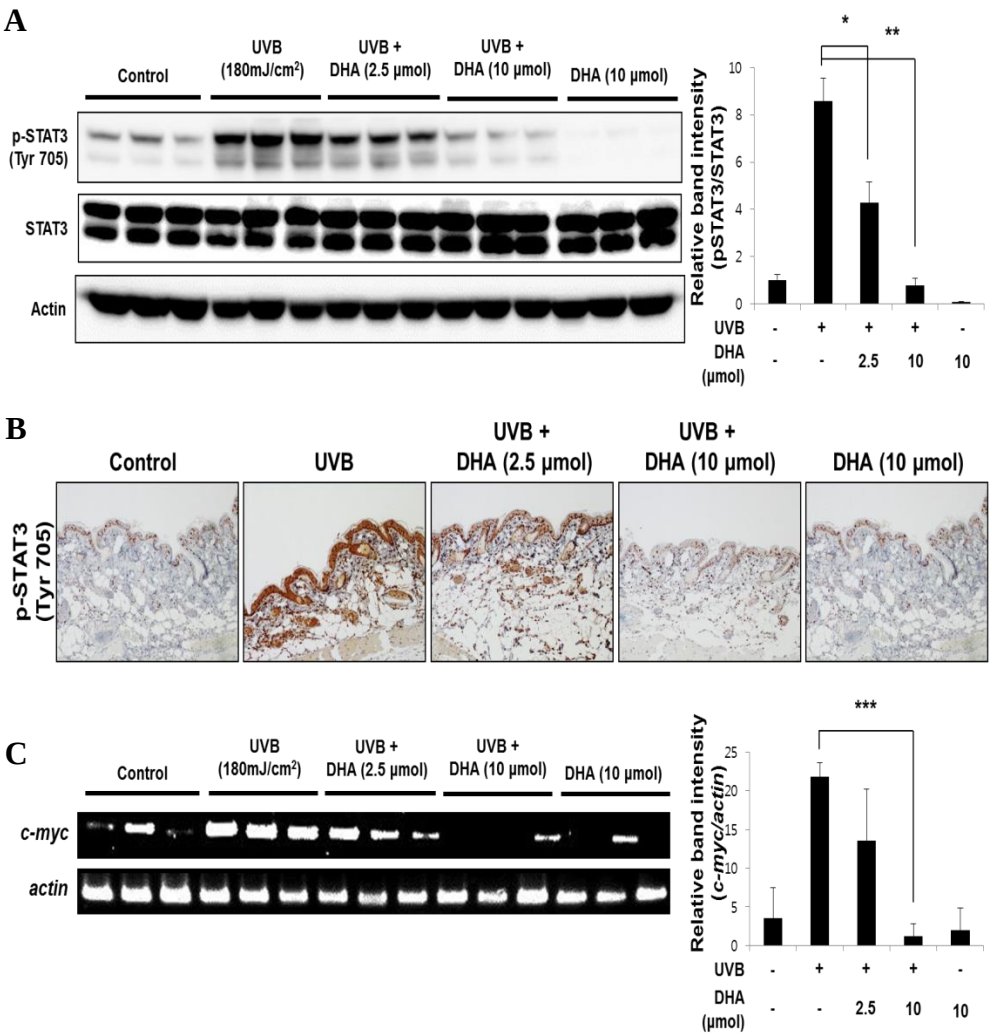

Figure 3. Effects of DHA on UVB-induced phosphorylation of STAT3 and expression of its target gene *c-myc* in the mouse skin. Animal treatment and other experimental conditions are same as described in the Figure 1 legend section. (A) Whole lysates (30 μg protein) from different treatment groups were separated by electrophoresis on 10% SDS-

polyacrylamide gel and immunoblotted to detect total and phosphorylated forms of STAT3 (Tyr^{705}). Quantification of p-STAT3 was normalized to that of STAT3 followed by statistical analysis of relative band density. (B) The sections of skin tissues were subjected to immunohistochemical analysis of phosphorylated of STAT3 at Tyr^{705}. Positive phosphorylation of STAT3 staining yielded a brown-colored product. Magnifications ×200. (C) Total RNA was isolated from differentially treated skin tissues using TRIzol® reagent according to the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to examine mRNA expression of *c-myc*. Quantification of *c-myc* was normalized to that of *actin* followed by statistical analysis of relative band density. Data are expressed as means ± SE. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

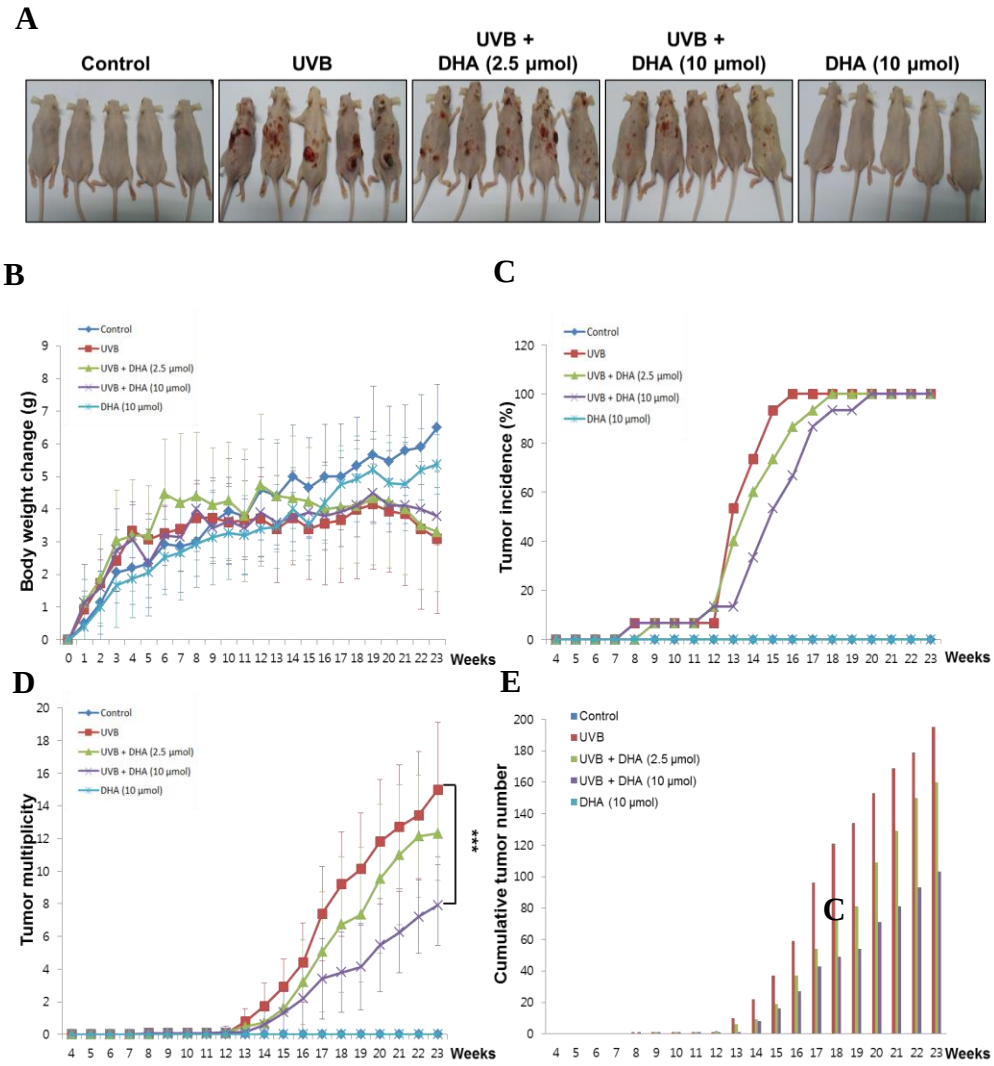

Figure 4. Effect of DHA on UVB-induced skin tumor development in HR-1 hairless mice. Female HR-1 hairless mice (*n* = 15 per treatment group) were topically treated on their backs with DHA (2.5 or 10 μmol) 30 min prior to UVB radiation (180 mJ/cm^2) three times a week until termination of

the experiment at 23rd week. (A) Representative phostographs of animals from different treatment groups, (B) The body weight change was measured on a weekly basis. Starting four week following UVB treatment, tumors of at least 1 mm diameter were monitored and counted every week till 23 weeks. The results were expressed as (C) the percentage of papilloma-bearing mice (incidence), (D) the average number of papillomas per mouse (multiplicity), and (E) the cumulative number of papillomas among tumor-bearing mice. Data are expressed as means ± SD. ***p <0.001.

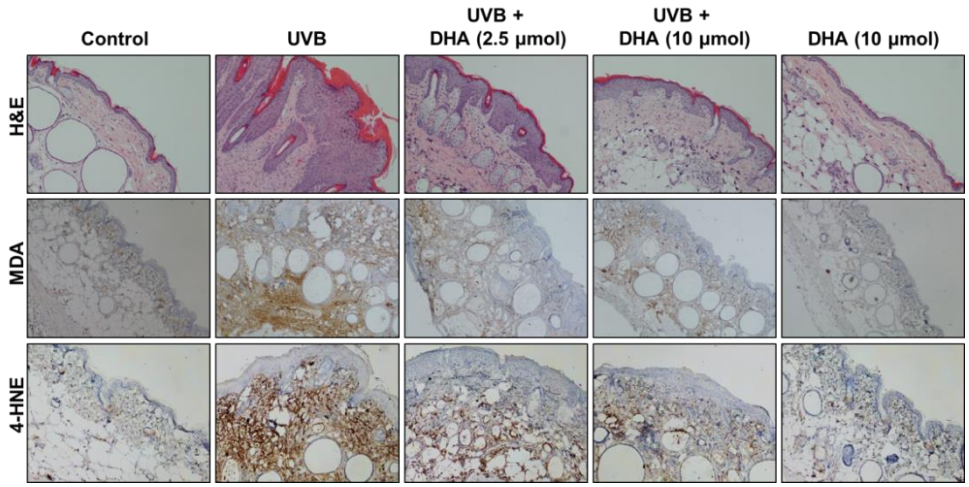

Figure 5. Effects of DHA on histopathologic features of mouse skin papillomas. H&E staining of papillomas showed hypertrophic squamous epithelium forming papillary fronds (upper panel). The respective sections were also immunostained for MDA (middle panel) and 4-HNE ((bottom panel), and counterstained with hematoxylin. Positive MDA and 4-HNE staining yielded a brown-colored product. Magnifications ×200 (H&E, 4-HNE), x100 (MDA).

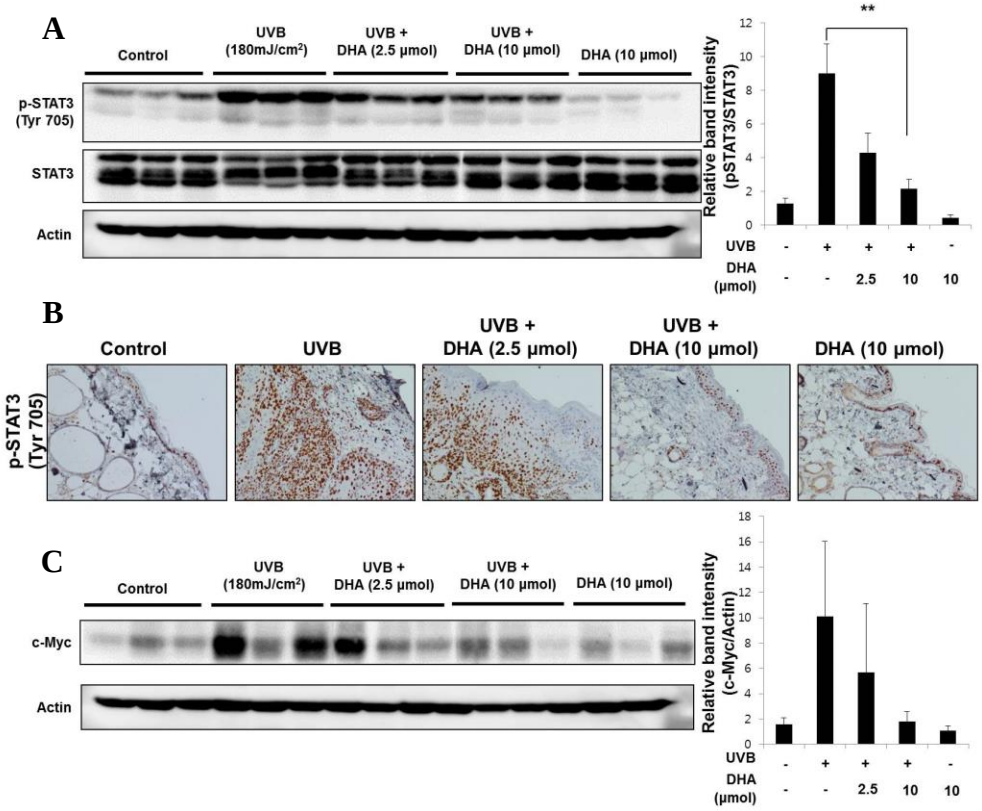

Figure 6. Effects of DHA on UVB-induced phosphorylation of STAT3 and expression of its target protein, c-Myc in the mouse skin papillomas. Animal treatment and other experimental conditions are described in the legend to Figure 3. (A) Whole surrounding tissue extracts (30 μg protein) were separated by electrophoresis on 10% SDS-polyacrylamide gel and immunoblotted to detect total and phosphorylated forms of STAT3 (Tyr^{705}). Quantification of pSTAT3 was normalized to that of STAT3 followed by statistical analysis of relative band density. Data are expressed as means ± SE. $^{**}p < 0.01$. (B) Formalin-fixed skin tissues pretreated with DHA and

exposed to UVB radiation were subjected to immunohistochemical analysis using a specific antibody to detect STAT3 phosphorylation at Tyr^{705}. Magnifications ×300. (C) Whole lysates (30 μg protein) were also analyzed for the protein expression of c-Myc by immunoblotting. Quantification of c-Myc immunoblot was normalized to that of actin followed by statistical analysis of relative band density.

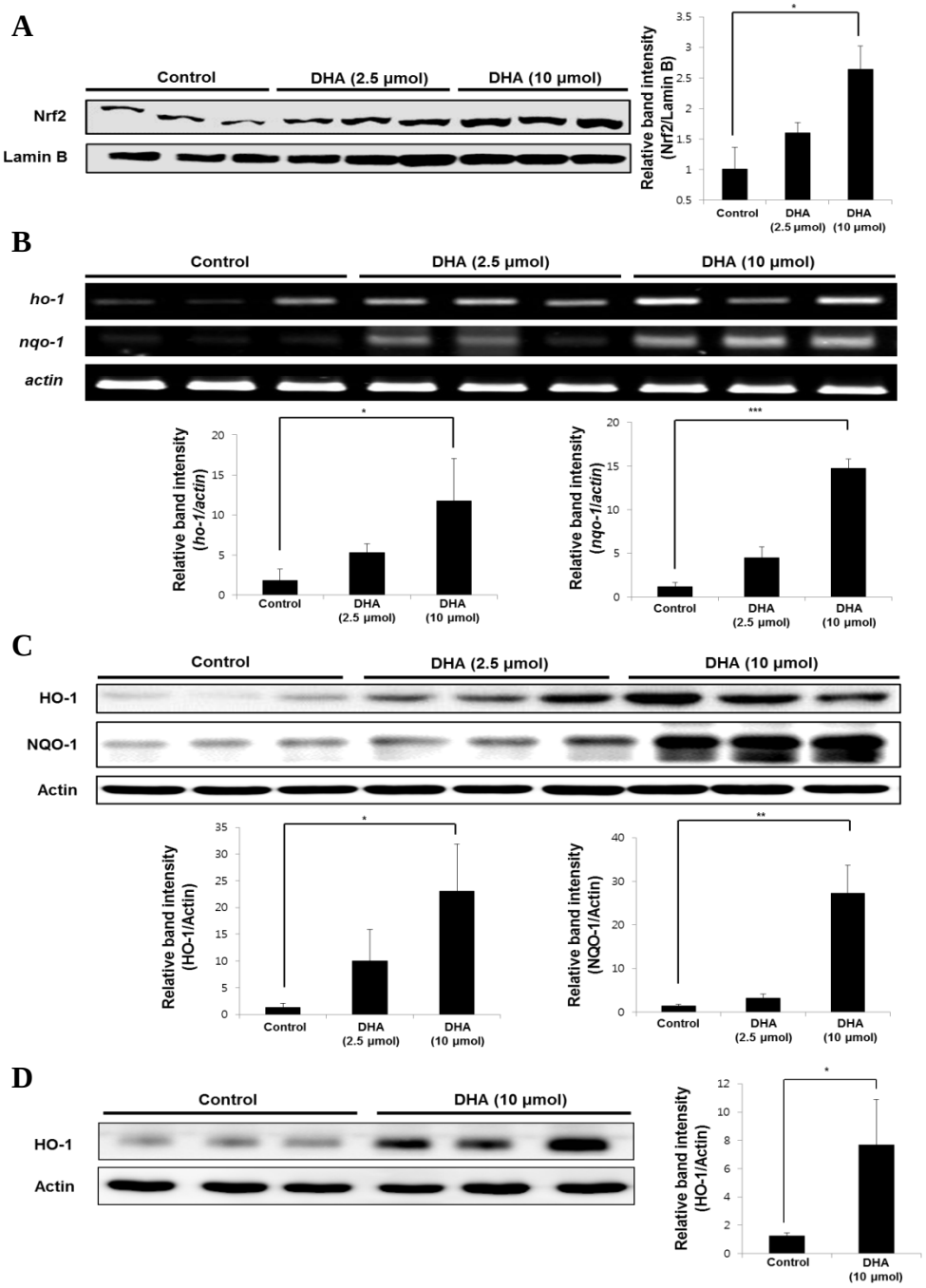

Figure 7. Effects of DHA on nuclear translocation of Nrf2 and the expression of HO-1 and NQO1 in HR-1 hairless mouse skin *in vivo*.

Dorsal skin of female HR-1 hairless mice (n = 5 per treatment group) were topically treated with DHA at the indicated doses. Control animals were treated with acetone in lieu of DHA. (A) After 2.5 h of treatment, the nuclear fraction was isolated and analyzed to determine the Nrf2 levels by western blotting. (B) Total RNA was isolated from skin tissues using TRIzol® reagent according to the manufacturer's protocol. RT-PCR analysis was performed to detect mRNA expression of *ho-1 and nqo-1*. Quantification of *ho-1 and nqo-1* was normalized to that of *actin* followed by statistical analysis of relative band density. (C) Epidermal tissue lysates (30 μg protein) were separated by electrophoresis on 10% SDS-polyacrylamide gel and immunoblotted to detect protein expression of HO-1. (D) After 23 weeks of treatment, whole tissue extracts (30 μg protein) were analyzed for the protein expression of HO-1 by immunoblotting. Quantification of HO-1 immunoblot was normalized to that of actin followed by statistical analysis of relative band density. Data are expressed as means ± SE. *p <0.05.

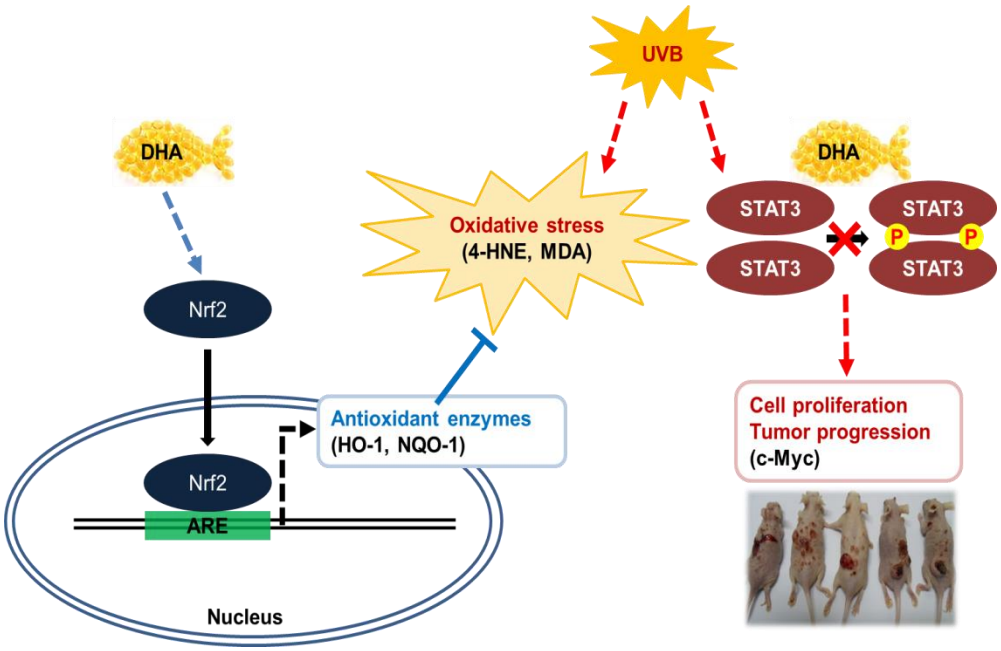

Figure 8. A proposed mechanism underlying the protective effects of DHA against UVB-induced inflammation and photocarcinogenesis in HR-1 hairless mouse skin

Discussion

The exposure to UVB radiation causes a variety of dermatologic disorders, such as inflammation, erythema, immunosuppression and cancer (Afaq et al. 2005, Halliday 2005, Matsumura and Ananthaswamy 2004). A wide variety of natural antioxidative and anti-inflammatory agents, in particular those present in our regular diet, have been shown to inhibit UVB-induced skin cancer (Mittal et al. 2003, Singh et al. 2006, Staniforth et al. 2006, Won et al. 2004). The ω-3 polyunsaturated fatty acids (PUFAs), present in fish oil and plant seed oil, possess a broad range of health beneficial properties such as antioxidant, anti-inflammatory and anti-cancer activities (Jho et al. 2004). DHA, a representative ω-3-PUFA, has been reported to inhibit UVB-induced oxidative and inflammatory responses in mouse skin (Rahman et al. 2011). Rahman *et al.* (2011) have demonstrated that topical application of DHA inhibited UVB-induced expression of NAD(P)H oxidase-4, an enzyme involved in the generation of ROS, and that of a pro-inflammatory enzyme cyclooxygenase-2 (COX-2) in hairless mouse skin. In line with study, we found that topical application of DHA attenuated epidermal hyperplasia and reduced the accumulation of lipid peroxidation products, such as MDA and 4-HNE in UVB-irradiated mouse skin. These findings are in good agreement

with the previously published antioxidative effects of the compound (Chen et al. 2013, Sunada et al. 2006).

Our study revealed for the first time that DHA treatment inhibited the incidence and the multiplicity of UVB radiation-induced mouse skin papillomagenesis. The inhibitory effects of DHA on the UVB-induced increase in MDA and 4-HNE levels in skin papillomas indicate that DHA suppress papilloma formation by virtue of its antioxidant effects in UVB-irradiated mouse skin.

Ahsan et al. (2005) have demonstrated that exposure to UVB radiation phosphorylate STAT3 at tyrosine 705 residue in SKH1 hairless mouse skin. Moreover, STAT3 activation promotes keratinocyte survival and proliferation in response to UVB radiation (Sano et al. 2005). In addition, Kim et al. (2009) reported that constitutive activation of STAT3 enhances UVB-induced skin carcinogenesis. Thus, inhibition of STAT3 signaling is considered as a pragmatic approach for the prevention of UVB-induced skin carcinogenesis. Our finding that DHA treatment diminished STAT3 phosphorylation at tyrosine 705 residue as well as the mRNA expression of its target gene product, *c-myc*, in acute or chronic UVB-irradiated mouse skin or papillomas, respectively, suggest that DHA exerts its skin cancer chemopreventive effects, at least in part, by blocking STAT3 activation.

The dimerization and subsequent transcriptional activation of STAT3

requires its phosphorylation at tyrosine-705 and serine-727 residues. While tyrosine phosphorylation of STAT3 is mediated largely by upstream Janus-activated kinases (Jak) (Aaronson and Horvath 2002), its serine phosphorylation is regulated by serine/threonine kinases (Turkson et al 1999, Zhang et al 2001). Among the serine/threonine kinase series of mitogen-activated protein (MAP) kinases, c-Jun-N-terminal kinase (JNK), but not extracellular kinase (ERK) or p38 MAP kinase, has been reported to regulate STAT3 serine phosphorylation in UVA-stimulated mouse epidermal (JB6) cells (Zhang et al 2001). Rahman et al. (Rahman et al 2011) reported that DHA inhibited UVB-induced phosphorylation of ERK and p38 MAP kinase in mouse skin. Thus, the effect of DHA on UVB-induced STAT3 phosphorylation at serine 727 residue merits further investigation.

Although UVB-induced ROS are involved in skin photodamage, body protects against oxidative stress through activation of a series of antioxidant and detoxification enzymes, collectively known as cytoprotective proteins. HO-1 and NQO1 are two representative cytoprotective proteins, which are transcriptionally regulated by a redox sensitive transcription factor Nrf2 (Surh et al 2008). Was et al. (Was et al 2011) demonstrated that mice expressing wild type HO-1 exhibited relatively reduced multiplicity of skin papillomas as compared to HO-1 knockout animals at the early stage of skin carcinogenesis. Long et al. (Long et al 2000, Long et al 2001) reported that

NQO1-null mice are more sensitive to chemically induced skin tumorigenesis as compared to wild type mice. Likewise, Nrf2 knockout mice are more susceptible to develop chemically induce skin tumors (Xu et al 2006). Since the induction of HO-1 and NQO1 elicit antioxidative and anti-inflammatory effects (Surh et al 2008), our findings that DHA increases the nuclear accumulation of Nrf2 and induces the expression of HO-1 and NQO1 in mouse skin may be attributed to its inhibitory effects on UVB-induced skin carcinogenesis.

In conclusion, the present study demonstrates that topical application of DHA protected against UVB-induced papillomagenesis by suppressing oxidative and inflammatory damages in mouse skin through the inhibition of lipid perdoxidation and downregulation of STAT3 activation **(Figure 8)**.

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국문초록

UVB 에 의해 유도된 마우스 피부발암과정에서 Docosahexaenoic Acid 의 암예방 효과

암의 발생과 진행에 있어서 지속적인 염증반응은 매우 중요한 연관성이 있는 것으로 알려져 있다. 자외선 종류 중 하나인 Ultraviolet B (UVB)는 피부에 노출 시 염증반응을 유도하는 것으로 보고되고 있다.

Docosahexaenoic acid (DHA)은 오메가-3 지방산 중의 대표적인 물질로 등푸른 생선 및 계란 등에 많이 포함되어 있다. 또한 DHA 는 일반적으로 뇌의 발달, 항 산화, 항 노화, 항 염증, 항암작용 등이 있는 것으로 알려져 있다.

본 연구자는 마우스 피부 암화과정에 대한 DHA 의 예방 효과를 알아보기 위해 UVB 로 유도된 마우스 피부염 모델과 피부암 모델을 이용하여 검토하였다.

암컷 HR-1 마우스에 DHA 를 Acetone 에 희석하여 2.5 μmol 와 10 μmol 두 가지 농도로 마우스 등에 도포한 후, 30 분 경과 후

UVB (180 mJ/cm^2) 를 쬐도록 하였다. 피부염의 모델에서 hematoxylin and eosin (H&E)로 염색하여 피부 조직을 분석한 결과 DHA 가 UVB 에 의해 유도된 염증적 조직손상을 감소시키는 것을 관찰하였다. 또한 대표적인 염증관련 lipidperoxidation marker 인 4-HNE 와 MDA 가 DHA 에 의해 그 수준이 감소됨을 확인 하였다. DHA 의 도포가 마우스 피부에서 UVB 에 의해 signal transducer and activator of transcription 3 (STAT3)의 활성화 및 이들 전사인자들의 조절을 받는 표적 단백질들의 발현을 경감시키는 것 또한 확인하였다.

피부암의 모델에서 DHA 의 예방효과를 관찰하기 위하여 본 연구자는 다음과 같이 실험하였다. 일주일에 3 번, 23 주 동안 암컷 HR-1 마우스에 DHA 를 2.5 μmol 와 10 μmol 두 가지 농도로 마우스 등에 도포한 후, 30 분 경과 후 UVB (180 mJ/cm^2) 를 쬐도록 하였다. UVB 는 HR-1 mouse 에서 유의적으로 papilloma 형성을 촉진하였으며 이는 DHA 에 의해 그 수가 감소하였다. 또한 H&E 염색을 통하여 많은 염증관련 세포들이 피부에 많이 침윤되었음을 확인 하였으며 피부염 모델에서와

마찬가지로 4-HNE, MDA 의 수준이 DHA 에 의해 감소되었고 STAT3 의 활성화도 경감되었다.

별도의 실험에서 DHA 단독 도포 시 항 산화 효소의 발현에 관여하는 nuclear factor-erythroid related factor-2 (Nrf2)의 수준이 증가됨을 확인 하였으며, Nrf2 전사인자가 유도하는 hemeoxygenase-1 (HO-1) 와 NAD(P)H quinoneoxidoreductase-1 (NQO1) 수준 또한 발현이 증가되었다.

이와 같은 결과들은 DHA 가 UVB 에 의해 유도된 STAT3 의 활성화를 경감시키고, 항 산화 효소의 발현을 유도하여 UVB 가 유도한 산화적 스트레스를 감소시켜 마우스의 피부암에 예방효과를 나타냄을 시사한다.

주요어 : UVB, 피부 암화, STAT3, DHA, 항산화 효소

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