Dietary grape seed proanthocyanidins inhibit UVB-induced oxidative stress and activation of mitogen-activated protein kinases and nuclear factor-κB signaling in *in vivo* SKH-1 hairless mice

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Abstract

We have shown previously that dietary grape seed proanthocyanidins (GSP) inhibit UVB-induced photocarcinogenesis in mice. As UVB-induced oxidative stress and oxidative stress–mediated signaling has been implicated in photocarcinogenesis, this study was designed to investigate the effect of dietary GSPs on UVB-induced oxidative stress in *in vivo* SKH-1 hairless mice. Here, we report that provision of dietary GSPs (0.2 and 0.5%, w/w) to mice exposed to either acute UVB irradiation (120 mJ/cm^2) or chronic irradiation of UVB inhibited depletion of glutathione peroxidase, catalase, and glutathione, and inhibited UVB-induced H_2O_2, lipid peroxidation, protein oxidation, and nitric oxide in mouse skin. As UV-induced oxidative stress mediates activation of mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways, we determined the effect of dietary GSPs on these pathways. We observed that dietary GSPs inhibited UVB-induced phosphorylation of extracellular signal-regulated kinase 1/2, c-Jun-NH_2-kinase, and p38 proteins of MAPK family, which seems to be mediated through reactivation of MAPK phosphatases. GSPs inhibited UVB-induced activation of NF-κB/p65 through inhibition of degradation of IκBα and activation of IκB kinase α (IKKα). As NF-κB–targeted genes play critical roles in inflammation and cellular proliferation, we assessed the effect of GSPs on proteins encoded by these genes. Dietary GSPs resulted in inhibition of the expression of proliferating cell nuclear antigen, cyclin D1, inducible nitric oxide synthase, and cyclooxygenase-2 in the skin. Collectively, our data show that GSPs have the ability to protect the skin from the adverse effects of UVB radiation via modulation of the MAPK and NF-κB signaling pathways and provide a molecular basis for the photoprotective effects of GSPs in an *in vivo* animal model. [Mol Cancer Ther 2007;6(3):995–1005]

Introduction

Human skin is exposed constantly to solar UV radiation. Overexposure of the skin to UV radiation results in the development of inflammation, oxidative stress, immune suppression, and DNA damage, which lead to several skin disorders, including hyperpigmentation, photoaging, or premature aging of the skin and melanoma and nonmelanoma (basal and squamous cell) skin cancers (1–3). The incidence of skin cancers, including both melanoma and nonmelanoma skin cancers, is growing as the population ages, as greater amounts of UV radiation reach the surface of the Earth because of depletion of the ozone layer, and as the use of sun tanning devices for cosmetic purposes increases.

The exposure of the skin to UV radiation induces the generation of reactive oxygen/nitrogen species or oxidative stress, which is capable of oxidizing lipids, proteins, or DNA. The oxidized products formed, including lipid hydroperoxides, protein carbonyls, or 8-hydroxydeoxyguanosine, have been implicated in the onset of skin cancers (4–6). Endogenous enzymatic and nonenzymatic antioxidant defense systems can protect the skin from UV-induced oxidative damage (7). There is considerable evidence that UV-induced oxidative stress mediates the phosphorylation of protein kinases through a series of cascades, such as mitogen-activated protein kinases (MAPK), and activation of transcription factors, such as nuclear factor-κB (NF-κB; refs. 8–11). Three structurally related but biochemically and functionally distinct MAPK signal transduction pathways have been identified in mammalian cells, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 (9, 10). The MAPKs belong to a family of serine/threonine protein kinases and are believed to act as a system that mediates signal transduction from the cell surface to the nucleus. They play a major role in triggering and coordinating gene responses in UV-induced damage. ERK1/2 is generally activated by mitogenic signals, whereas JNK and p38 are primarily activated by environmental stresses, such as UV radiation, inflammatory

Received 10/26/06; revised 12/26/06; accepted 2/1/07.

Grant support: National Cancer Institute/NIH grant CA104428-01 and the Veterans Administration Merit Review Award (S.K. Katiyar).

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doi:10.1158/1535-7163.MCT-06-0661

cytokines, heat shock, and DNA-damaging agents (8–10). Activation of ERKs stimulates proliferation and differentiation and also plays a role in tumor promotion, especially tumorigenesis stimulated by the oxidative state (12). Phosphorylation of JNK and p38 plays a role in cellular differentiation and inflammatory responses (12, 13). Antioxidants have been shown to attenuate the activation of MAPK signaling (11, 12, 14), thereby indicating that MAPK signaling pathway is an important target of oxidative stress. It has been documented that NF-κB is a downstream target of the MAPK signal transduction pathway, and that activation of NF-κB plays crucial roles in inflammation, cellular proliferation, and induction of cancers. Therefore, the signaling pathways leading to the regulation of NF-κB activity have become targets for chemopreventive and chemotherapeutic approaches.

Grapes (*Vitis vinifera*) are one of the most widely consumed fruits in the world. They are rich in polyphenols, with ~60% to 70% of grape polyphenols being found in the seeds. These polyphenols are found in the form of catechin monomers, or their dimers, trimers, and oligomers, and are commonly known as proanthocyanidins. Grape seed proanthocyanidins (GSP) are a mixture of several polyphenols/flavanols and mainly contain proanthocyanidins (89%), as has been described previously (15). We have shown previously that dietary GSPs inhibit UV radiation–induced skin cancer in mice (15) and consider that the combined photoprotective effect of GSPs should be better than any individual component because of their synergistic effects against the adverse effects of UV irradiation. We have shown previously that *in vitro* treatment of normal human epidermal keratinocytes with GSPs inhibits UV-induced oxidative stress and oxidative stress–mediated activation of MAPK and NF-κB cellular signaling pathways (16). The present study was designed to investigate the relevance of these *in vitro* findings to the *in vivo* situation in SKH-1 hairless mice. We found that dietary GSPs can inhibit the adverse effects of both acute and chronic UV exposure of SKH-1 hairless mice, including (*a*) inhibition of depletion of endogenous antioxidant defense enzymes, such as glutathione peroxidase (GPx), glutathione (GSH), and catalase; (*b*) suppression of oxidative stress in terms of hydrogen peroxide and nitric oxide production, and lipid and protein oxidation; (*c*) inhibition of activation of the MAPK and NF-κB signaling pathways; and (*d*) inhibition of expression of NF-κB–responsive genes.

Materials and Methods

Chemicals and Antibodies

Antibodies for phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}), JNK (Thr^{183}/Tyr^{185}), p38 (Thr^{180}/Tyr^{182}); nonphosphorylated ERK1/2, JNK, and p38; and the anti–β-actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies for NF-κB, IκBα, IKKα, inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), cyclin D1, proliferating cell nuclear antigen (PCNA); ERK1/2, JNK, and p38 MAPK phosphatases (MKP-1, V-15)–specific antibody; and the anti-mouse IgG horseradish peroxidase–linked and anti-rabbit IgG horseradish peroxidase–linked secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The protein assay kit was obtained from Bio-Rad (Hercules, CA), and the enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals used in this study were of analytic grade and purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

The 6- to 7-week-old female SKH-1 hairless mice used in this study were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed five per cage and acclimatized for at least 1 week before start of the experiment, and housed in the Animal Resource Facility of the University of Alabama at Birmingham under the following conditions: 12 h dark/12 h light cycle, 24 ± 2°C temperature, and 50 ± 10% relative humidity. The mice were fed a standard AIN76A diet (Harlan Teklad, Madison, WI) with or without GSPs (0.2% or 0.5%, w/w) and water *ad libitum*. The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

The GSP extract of grape seeds were obtained from the Kikkoman Corporation (Tokyo, Japan) and the chemical composition has been described earlier (15). Experimental diets containing GSPs (0.2% or 0.5%, w/w) were prepared separately in AIN76A powdered control diet, as detailed previously (15). The GSPs were given in the diet of the mice at least 2 weeks before UVB irradiation and continued until the termination of the experiment.

UVB Light Source and Irradiation Protocol

SKH-1 hairless mice were exposed to UVB as described previously (15). Briefly, the dorsal skin area of the mice was exposed to UVB radiation from a band of four UVB lamps (Daavlin, UVA/UVB Research Irradiation Unit, Bryan, OH) equipped with an electronic controller to regulate UV dosage at the fixed distance of 22 cm from the lamps to the dorsal skin surface of the mice. The majority of the resulting wavelengths were in the UVB (290–320 nm; >80%) and UVA (<20%) range with peak emission at 314 nm as monitored. Mice were either exposed to acute UVB dose (120 mJ/cm^2) or to chronic UVB exposures. For chronic UVB exposures, mice were exposed to UVB (120 mJ/cm^2) thrice a week for 1 month.

Preparation of Cytosols and Microsomal Fractions

Twenty-four hours after the last UVB exposure, mice were sacrificed and skin samples were collected. The epidermal layer was separated from the whole skin and then cytosolic and microsomal fractions were prepared as described previously (17). Briefly, epidermis was homogenized with a Polytron System, PT 3100 (Kinematica, Lucerne, Switzerland) in PBS buffer containing potassium chloride (1.1%, w/v) and centrifuged at 18,000 × *g* for 15 min at 4°C to prepare cytosolic and microsomal fractions (17).

Assays for Antioxidant Enzymes and GSH

The levels of GPx, GSH, and catalase were measured in cytosolic fractions following the standard analytic methods of Flohe and Gunzler (18), Akerboom and Sies (19), and Nelson and Kiesow (20), respectively. The experiments were repeated at least thrice.

Quantitative Analysis of Reactive Oxygen Species

The release of reactive oxygen species (ROS), including H_2O_2 by epidermal cells, was determined using dihydrorhodamine 123 as a specific fluorescent dye probe as described previously (1, 16). Epidermal single-cell suspensions were prepared as described (1, 17), and cells were subjected to the analysis of ROS (1, 17). Briefly, 1 million cells from different treatment groups were taken in each well of a 24-well tissue culture plate in duplicate. These cells were treated with dihydrorhodamine (5 μmol/L) for 45 min. Reduced dihydrorhodamine is irreversibly oxidized and converted to the red fluorescent compound rhodamine 123 by UVB-induced release of ROS. Fluorescence intensity was recorded on a Synergy HT (Bio-TEK Instruments, Inc., Highland Park, VT) fluorescence plate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Histochemical Detection of H_2O_2-Producing Cells

Immunohistochemical detection of H_2O_2 in control and UV-irradiated skin was done following a previously described procedure (1). Briefly, 6-μm-thick skin sections were incubated with 0.1 mol/L Tris-HCl buffer (pH 7.5), containing 1 mg/mL glucose and 1 mg/mL diaminobenzidine for 6 h at 37°C. Sections were then washed in distilled water and counterstained with methyl green.

Assay for Nitric Oxide

The levels of nitric oxide in the skin were determined by measuring their stable degradation products, nitrate and nitrite, using a colorimetric Nitric Oxide Assay kit (Oxford Biomedical Research, Inc., Oxford, MI) following the manufacturer's protocol. In this method, nitrate is enzymatically converted into nitrite by the enzyme nitrate reductase followed by determination of nitrite using Griess reagent.

Quantitative Analysis of Oxidized Proteins

The quantitative analysis of protein oxidation in terms of the formation of protein carbonyls was done using 2,4-dinitrophenylhydrazine as described previously (21). Briefly, the nucleic acids present in the skin lysates that contain carbonyl groups reactive with 2,4-dinitrophenylhydrazine were initially precipitated out using streptomycin sulfate followed by dialysis against water for 2.5 h. The protein carbonyl content was calculated from the molar absorption coefficient (ε) of 22,000 $(mol/L)^{-1}$ cm^{-1}.

Assay for Lipid Peroxidation

The epidermal microsomal fraction was used to determine the epidermal lipid peroxidation level using the thiobarbituric acid reaction method, as described previously (15). Briefly, 0.2 mL of the microsomal fraction was treated with 0.2 mL of 8.1% SDS and 3 mL thiobarbituric acid. Total volume was made up to 4 mL with distilled water and kept at 95°C in a water bath for 1 h. Color was extracted with *n*-butanol and pyridine (15:1 v/v). The absorbance was measured at 530 nm, and the resultant lipid peroxidation was expressed in terms of percentage of control.

Immunostaining of Phosphorylated MAPK Proteins

Skin samples were collected and frozen immediately in liquid nitrogen after embedding in optimal cutting temperature medium. The frozen skin samples were stored at −80°C for further use. Immunoperoxidase staining was done to detect the expression of phosphorylated proteins of MAPK family on a per-cell level. Briefly, frozen skin sections (6 μm thick) were fixed in cold acetone and nonspecific staining was blocked with 5% goat serum in PBS buffer. Thereafter, sections were incubated with phosphospecific ERK1/2, JNK, or p38 antibodies for 2 h at room temperature. After washing in PBS, endogenous peroxidase in skin sections was blocked using 0.5% H_2O_2 in PBS. Sections were further incubated with biotinylated goat anti-rabbit secondary antibody and Vector ABC Elite peroxidase kit reagents according to the manufacturer's protocol. After washing in PBS buffer, sections were incubated with diaminobenzidine substrate solution with peroxidase enzyme and counterstained with either methyl green (1%, w/v) or hematoxylin (for p38 counterstaining). Representative sections were selected for the presentation from samples from five mice in each group.

Western Blot Analysis and Densitometry

The epidermis was separated from the whole skin as described previously (1, 22) and was used for the preparation of epidermal lysates or nuclear fractions (16). The proteins (25–50 μg) were resolved over 8% to 12% SDS-PAGE gels and transferred onto a nitrocellulose membrane. After blocking the nonspecific binding sites in blocking buffer [5% nonfat dry milk, 1% Tween 20 in 20 mmol/L TBS (pH 7.6)], the blots were then incubated overnight with specific primary antibodies to be assessed. The blot was washed and incubated again with appropriate horseradish peroxidase–conjugated secondary antibody, and the protein expression was detected and visualized using an enhanced chemiluminescence detection system (Amersham Life Sciences, Arlington, IL).

The density of each band in an immunoblot was analyzed using the Scion Image Program (NIH, Bethesda, MD). The relative numerical values are shown under each immunoblot. The values for the control group (non–UVB exposed) were assigned the value 1 (arbitrary unit), and comparison was then made with densitometry values of other treatment groups. To ensure equal protein loading, the membranes were stripped and reprobed with anti–β-actin antibodies using the protocol described above.

ELISA for NF-κB/p65

For quantitative analysis of NF-κB/p65, Trans^{AM} ELISA kit (Active Motif, Carlsbad, CA) was used following the manufacturer's protocol. For this assay, the nuclear extracts of epidermal skin samples from various treatment groups were prepared using the Nuclear Extraction kit (Active Motif) according to the manufacturer's direction.

Absorbance was recorded at 450 nm with reference taken at 650 nm. The assay was done in duplicate and the results are expressed as the percentage absorbance of control (non-UVB exposed) group.

Results

Dietary GSPs Prevent UVB-Induced Depletion of GSH, GPx, and Catalase in the Skin

It is well established that exposure of skin to UV radiation results in depletion of the antioxidant defense capabilities at the UV-irradiated site (1, 17, 23). Under the conditions used in our experiments, acute UV exposure resulted a 24% reduction in the levels of reduced GSH in exposed skin compared with the levels in the skin of non-UVB exposed (control) mice, with chronic exposure resulting in a 56% reduction (Fig. 1A) when measured 24 h after the last exposure. The depletion of reduced GSH levels in response to acute irradiation were reduced by 50% ($P < 0.05$) on provision of 0.2% (w/w) GSPs in the diet and 83% ($P < 0.001$) on provision of 0.5% (w/w) GSPs in the diet. Similarly, the depletion of GSH levels in response to chronic irradiation was reduced by provision of GSPs in the diet, with a 45% reduction ($P < 0.05$) on provision of 0.2% GSPs in the diet and a 66% reduction on provision of 0.5% GSPs in the diet.

UV irradiation resulted in depletion of the GPx at the site of irradiation, with acute irradiation resulting in a 16% depletion and chronic irradiation resulting in a 42% ($P < 0.001$) depletion (Fig. 1B). In this case of GPx, the depletion in response to acute irradiation was reduced by 69% ($P < 0.05$) on provision of 0.2% (w/w), and 100% ($P < 0.001$) on provision of 0.5% (w/w) GSPs in the diet. The depletion of GPx levels in response to chronic irradiation also was reduced by provision of GSPs in the diet, with a 38% reduction ($P < 0.01$) on provision of 0.2% and a 76% reduction ($P < 0.005$) on provision of 0.5% GSPs in the diet (Fig. 1B).

Catalase has a role in the catalytic conversion of H_2O_2 to oxygen and water and thus contributes to reductions in the levels of oxidative stress. UV irradiation resulted in significant depletion of catalase at the site of irradiation, with acute irradiation resulting in a 34% depletion ($P < 0.05$) and chronic irradiation resulting in a 65% depletion ($P < 0.001$; Fig. 1C). The depletion of catalase levels in response to acute irradiation was reduced by 20% ($P < 0.05$) on provision of 0.2% (w/w) and by 47% ($P < 0.01$) on provision of 0.5% (w/w) GSPs in the diet. The depletion of catalase levels in response to chronic irradiation also was reduced by provision of GSPs in the diet, with a 41% reduction ($P < 0.01$) on provision of 0.2% (w/w) GSPs in the diet and a 77% reduction ($P < 0.005$) on provision of 0.5% (w/w) GSPs in the diet (Fig. 1C).

Dietary GSPs Inhibit UVB-Induced Release of ROS in Epidermal Cells

Under the conditions of our experiments, acute exposure of mouse skin to UVB resulted in a 3-fold ($P < 0.01$) enhancement of the intracellular release of ROS, including H_2O_2 in the epidermal cells and chronic exposure resulted in a 4.8-fold ($P < 0.001$) enhancement compared with the epidermal cells from non–UVB-exposed control mice. Administration of dietary GSPs (0.2% and 0.5%, w/w)

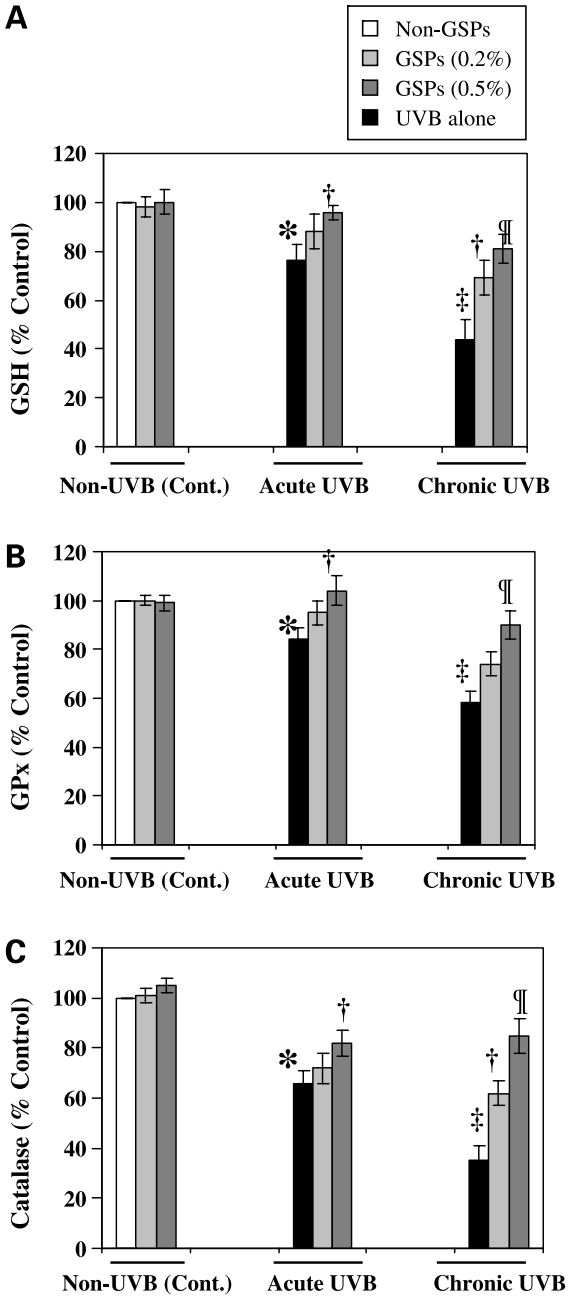

Figure 1. Dietary GSPs inhibit UVB-induced depletion of reduced GSH (**A**), GPx (**B**), and catalase (**C**) in SKH-1 hairless mouse skin. Mice were irradiated either with an acute UVB dose of 120 mJ/cm^2 or with UVB (120 mJ/cm^2) on alternate days for 1 mo. Mice were sacrificed 24 h after the last UVB exposure. Epidermal skin samples were collected, and cytosolic fractions were analyzed for the levels of antioxidant enzymes, as described in Materials and Methods. *Columns,* mean ($n = 5$); *bars,* SD. Significant difference versus control (non–UVB-exposed): *, $P < 0.05$; ‡, $P < 0.001$. Significant prevention versus UVB-exposed group: †, $P < 0.01$; ¶, $P < 0.005$.

significantly inhibited the UVB-induced intracellular release of ROS in epidermal cells in both acute and chronic UVB-exposed mice ($P < 0.01$; 0.005; Fig. 2A1). We also detected the release of H_2O_2, a marker of oxidative stress, through the use of histochemical staining of $H_2O_2^+$ cells in the skin of control mice, mice that had been chronically exposed to UVB irradiation and mice that had received a GSP-supplemented diet during chronic exposure to UVB irradiation. The $H_2O_2^+$ cells were not detectable in the skin of control mice (Fig. 2A2), whereas H_2O_2-producing cells were clearly apparent in the UVB-exposed skin. As shown in Fig. 2A2, dietary GSPs markedly reduced the number of H_2O_2-producing cells in the epidermis of mice that were chronically exposed to UVB irradiation.

Dietary GSPs Prevent UVB-Induced Oxidation of Lipids and Proteins in the Skin

One of the hallmarks of UV-induced oxidative stress is the formation of oxidized macromolecules, including lipid peroxidation. We therefore used lipid peroxidation as a marker of photo-oxidative damage. We found that both acute and chronic exposure of mouse skin to UVB resulted in increased lipid peroxidation (Fig. 2B). When mice were exposed to an acute UV exposure of 120 mJ/cm^2, the level of lipid peroxidation was increased to 140% ($P < 0.05$) of the levels in control (non-UV exposed) mice and chronic exposure led to an even greater increase of 212% ($P < 0.001$) of the levels in the control mice. Administration of GSPs (0.2% and 0.5%) as a supplement to the control diet resulted in significant inhibition of the levels of lipid peroxidation induced by acute (45–75%, $P < 0.01$–0.005) or chronic UVB exposure (32–69%, $P < 0.01$–0.005).

Oxidation of some amino acid residues of proteins leads to the formation of carbonyl derivatives that affects the nature and function of proteins (24). The presence of carbonyl groups has been used as a measure of oxidative damage of proteins under conditions of oxidative stress (25). We analyzed UV-induced protein carbonyl formation as a measure of oxidative damage in the mouse skin. We found that acute and chronic UV exposure of the mouse skin resulted in a 4- and 10-fold increase in the level of protein carbonyls compared with non–UV-exposed control mice when measured at 24 h after UV exposure (Fig. 2C). The increase in protein carbonyls in response to acute irradiation was reduced by 33% ($P < 0.01$) on provision of 0.2% and by 70% ($P < 0.005$) on provision of 0.5% GSPs in the diet. The increase in protein carbonyls in response to chronic irradiation also was reduced by provision of GSPs in the diet, with a 42% reduction ($P < 0.01$) on provision of 0.2% (w/w) GSPs in the diet and a 65% reduction ($P < 0.005$) on provision of 0.5% (w/w) GSPs in the diet (Fig. 2C).

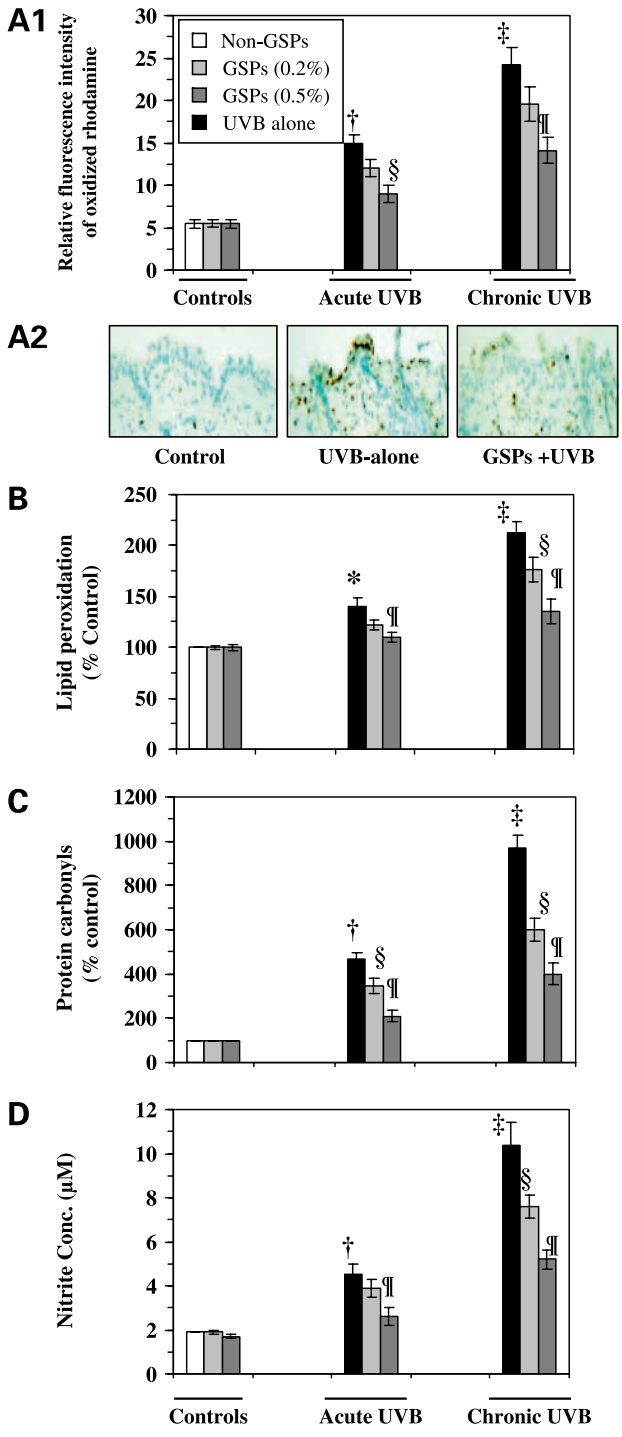

Figure 2. Dietary GSPs inhibit UVB-induced oxidative stress in the mouse skin in terms of total ROS (**A1**), H_2O_2-producing cells (**A2**), lipid peroxidation (**B**), protein oxidation (**C**), and nitric oxide generation (**D**). Mice were treated as described in Fig. 1. Epidermal single-cell suspensions were used to determine the release of H_2O_2 using dihydrorhodamine 123 as a fluorescence dye probe, as described in Materials and Methods (**A1**). Immunohistochemical detection of H_2O_2-producing cells is apparent as dark brown staining (**A2**). A representative micrograph is shown from each group ($n = 5$). Epidermal cytosolic fractions were analyzed for the determination of nitric oxide in the form of stable nitrite and protein carbonyls, and microsomal fractions were analyzed for lipid peroxidation. Lipid peroxidation and protein carbonyls are expressed in terms of percentage of control (non–UVB-exposed skin). *Columns,* mean ($n = 5$); *bars,* SD. Significant difference versus control (non–UVB-exposed): *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$. Significant prevention versus UVB-exposed groups: §, $P < 0.01$; ¶, $P < 0.005$.

Dietary GSPs Inhibit UVB-Induced Nitric Oxide Production

Nitric oxide was determined in the form of its stable product, nitrite. Quantitative analysis of NO reveals that exposure of skin to UVB induces NO production by >2- and 5-fold, respectively, after an acute and chronic exposure of UVB (Fig. 2D) compared with control mice that were not exposed to UVB radiation. Administration of dietary GSPs at the concentration of 0.2% and 0.5% (w/w) significantly inhibited UVB-induced production of nitric oxide in the skin under conditions of both acute (23% and 65%, $P < 0.005$) and chronic (33% and 59%, $P < 0.05$ and $P < 0.005$) UVB exposure.

Dietary GSPs Inhibit UVB-Induced Phosphorylation of MAPK Proteins

UVB-induced oxidative stress has been implicated in phosphorylation of MAPK proteins; thus, we determined the effect of dietary GSPs on UVB-induced phosphorylation of the ERK1/2, JNK, and p38 proteins of MAPK family in mouse skin using Western blot analysis. Western blotting and subsequently analysis of intensity of bands relative to β-actin indicated that exposure of mice with acute UVB dose enhanced the phosphorylation of ERK1/2 protein compared with non–UVB-exposed mouse skin (Fig. 3, *left*). However, administration of dietary GSPs (0.2 and 0.5%, w/w) inhibited acute UVB irradiation–induced phosphorylation of ERK1/2 in the mouse skin compared with non–GSP-treated but UVB-exposed mice. The inhibitory effect of dietary GSPs at the dose of 0.5% in the diet on UVB-induced phosphorylation of ERK1/2 was greater than the effect of the dose of 0.2% GSPs. Using a similar approach, we found that dietary GSPs at the doses of 0.2% and 0.5% (w/w) resulted in inhibition of acute UVB-induced phosphorylation of JNK and p38 proteins. It is also clearly apparent that the provision of 0.5% GSPs in the diet is more photoprotective than provision of 0.2% of GSPs in the diet. Importantly, treatment of mice with dietary GSPs alone at the levels of 0.2% or 0.5% (w/w) did not induce the phosphorylation of ERK1/2, JNK, or p38 proteins of MAPK family (data not shown). Further, the total amount of ERK1/2, JNK, and p38 proteins remain unchanged in each treatment group.

We also examined the effect of dietary GSPs against chronic exposure of mice with UVB radiation (Fig. 3, *right*). Western blot analysis revealed that administration of dietary GSPs (0.2% and 0.5%) markedly inhibited chronic UVB-induced phosphorylation of ERK1/2, JNK, and p38 proteins, with the photoprotective effect of 0.5% GSPs being >0.2% GSPs in diet. It was evident from the data obtained that the degree of inhibition of UVB-induced phosphorylation of MAPK proteins was greater in acute UVB-exposed mice than that in chronic UVB-exposed mice. Further, these changes were not due to differences in the amounts of proteins loaded on the gels as equivalent protein loading was confirmed by probing stripped blots for β-actin (Fig. 3, *right*).

To further confirm our results obtained using Western blot analysis, we did immunohistochemical examination of skin samples to determine phosphorylation of MAPK proteins. As shown in Fig. 4, the intensity of staining and number of ERK1/2-, JNK-, and p38-positive cells were higher in UVB-exposed skin compared with the skin of control non–UVB-exposed mouse skin. Administration of dietary GSPs (0.5%) resulted in inhibition of UVB-induced epidermal phosphorylation of ERK1/2 compared with non–GSP-treated UVB-exposed animals. The intensity of staining of pERK1/2-postive cells was markedly lower in GSP-treated mice compared with non–GSP-treated but UVB-exposed mice. Similarly, dietary GSPs also inhibited UVB-induced phosphorylation of JNK and p38 proteins (Fig. 4). These observations on a per-cell level after immunostaining further confirmed our observations obtained through Western blot analysis.

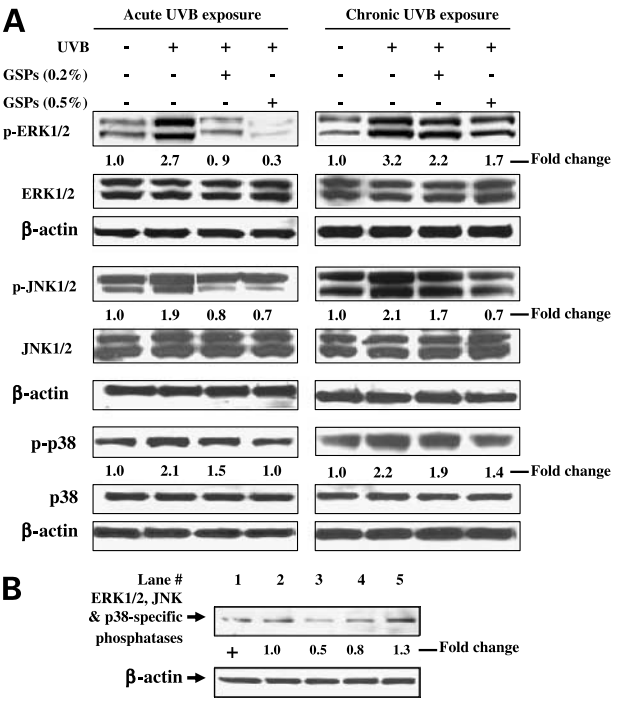

Figure 3. Dietary GSPs inhibit acute (*left*) or chronic (*right*) UVB-induced phosphorylation of MAPK proteins in SKH-1 hairless mouse skin (**A**). Mice were treated as described in Fig. 1. Epidermal skin lysates were prepared to determine the phosphorylated and total protein levels of ERK1/2, JNK, and p38 using Western blot analysis. A representative blot is shown from three independent experiments with identical observations, and equivalent protein loading was confirmed by probing stripped blots for β-actin. The relative density of each band in phosphorylated MAPK immunoblots is shown under each immunoblot as a fold-change compared with non–UVB-exposed control, which has been assigned the arbitrary unit 1. **B**, dietary GSPs prevent chronic UVB-induced oxidative stress–mediated depletion of the level of MAPK phosphatases. The density of each band was determined relative to the bands of β-actin and is shown under the immunoblot as a fold change compared with non–UVB-exposed control, which has been assigned the arbitrary unit 1. Treatment groups: *lane 1,* positive control of phosphatases from human epidermoid carcinoma A431 cells; *lane 2,* control skin (non-UVB); *lane 3,* UVB-exposed; *lane 4,* UVB+ GSPs (0.2%, w/w); *lane 5,* UVB+ GSPs (0.5%, w/w).

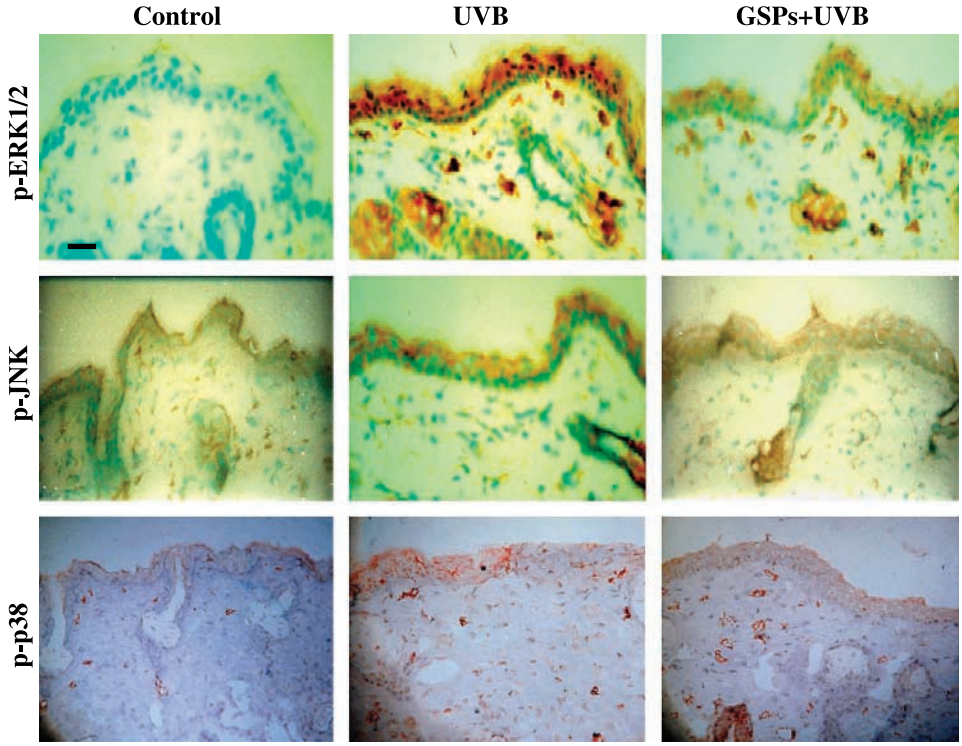

Figure 4. Dietary GSPs inhibit chronic UVB exposure–induced phosphorylation of ERK1/2 (*top*), JNK (*middle*), and p38 (*bottom*) proteins of MAPK family in SKH-1 hairless mouse skin. Mice were chronically UVB exposed as described in Fig. 2. The 5-μm-thick sections of frozen skin were analyzed for UVB-induced phosphorylation of ERK1/2, JNK, and p38 proteins after immunoperoxidase staining, as described in Materials and Methods. The intensity of the staining and the number of UVB-induced phosphorylated ERK1/2- and JNK-positive cells appear dark brown, whereas phosphorylated p38-positive cells appear red ($n = 5$); magnification, ×40. Representative examples of micrographs of staining from each group.

Dietary GSPs Inhibit or Restore UVB-Induced Depletion of MAPK Phosphatases

To determine the negative regulation of MAPK phosphatases on UVB-induced increases in the phosphorylation of MAPK proteins, we determined the levels of ERK1/2, JNK, and p38-specific MAPK phosphatases in epidermal skin lysates using Western blot analysis. The antibodies against MAPK phosphatases (MKP-1) have dual specificity directed to phosphothreonine and phosphotyrosine residues within MAPKs. Western blotting and subsequent analysis of the density of bands relative to β-actin revealed that chronic UVB exposure suppressed the levels of MAPK phosphatases (MKP-1) specific to MAPK proteins (e.g., ERK, JNK, and p38) in the mouse skin (Fig. 3B, *lane 3*) compared with non–UVB-exposed control mice (Fig. 3B, *lane 2*); however, the UVB-induced suppression or inactivation of MAPK phosphatases was restored in mice that were treated with dietary GSPs at the level of 0.2% (lane 4) and 0.5% (lane 5), as shown in Fig. 3B.

Dietary GSPs Inhibit UVB-Induced Activation of NF-κB/p65 via Their Inhibitory Effect on IKKα and the Degradation of IκBα in Mouse Skin

NF-κB/p65 is a downstream target of the MAPK signal transduction pathways. Our Western blot analysis indicated that chronic exposure of mice to UVB resulted in markedly greater activation of NF-κB/p65 and its translocation to the nucleus than non–UVB-exposed control mice. This finding was consistent with our data that indicate that the level of oxidative stress and phosphorylation of MAPK proteins are higher in UVB-exposed mice than control mice. However, the treatment of mice with dietary GSPs (0.2% and 0.5%) resulted in inhibition of UVB-induced activation and translocation of NF-κB/p65 to the nucleus compared with non–GSP-treated but UVB-exposed mice (Fig. 5A). The inhibitory effect of GSPs on NF-κB activation at a dose of 0.5% in diet was >0.2%. The inhibitory effect of dietary GSPs was further confirmed using ELISA for analysis of NF-κB (Fig. 5B). The administration of dietary GSPs at the doses of 0.2% and 0.5% in the control diet significantly inhibited chronic UVB-induced activation and translocation of NF-κB/p65 to the nucleus by 58% ($P < 0.01$) and 92% ($P < 0.001$), respectively.

Chronic UVB exposure also resulted in activation of IKKα in the skin compared with non–UVB-exposed control mice. The induction of IKKα has been shown to be essential for UVB-induced phosphorylation and degradation of IκBα. Western blot analysis indicated that the activation level of IKKα was higher in the skin of UVB-irradiated mice; however, administration of dietary GSPs inhibited the activation levels of IKKα in cytosols compared with non–GSP-treated but UVB-exposed mice (Fig. 5C). In contrast, degradation of IκBα was noted in UVB-irradiated mice compared with non–UVB-irradiated mice, whereas supplementation of GSPs with control diet at the levels of 0.2% and 0.5% (w/w) inhibited UVB-induced degradation of IκBα.

Dietary GSPs Inhibit the Expression of NF-κB–Targeted Proteins in UVB-Exposed Skin

We next examined whether dietary GSPs have the ability to inhibit the induction of NF-κB–responsive proteins, such as PCNA, cyclin D1, iNOS and COX-2, in chronic UVB-exposed SKH-1 hairless mouse skin. As shown in Fig. 6,

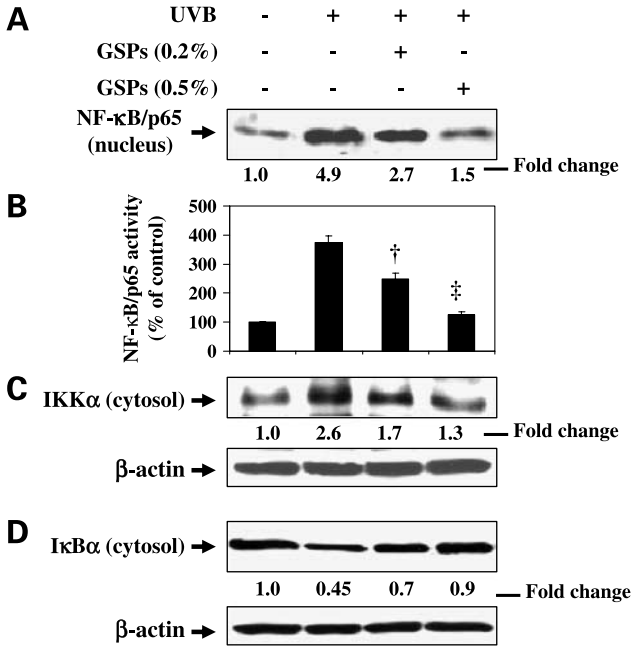

Figure 5. Dietary GSPs inhibit chronic UVB irradiation–induced activation of NF-κB/p65 and IKKα, and degradation of IκBα in SKH-1 hairless mouse skin. Mice were treated as described in Fig. 1. The activation of NF-κB/p65 (**A**), IKKα (**C**), or degradation of IκBα (**D**) was determined using Western blot analysis. The activity of NF-κB in nuclear fraction of skin lysates was measured using ELISA (**B**). A representative blot is shown from three independent experiments with almost identical observations ($n = 5$). Equivalent protein loading was confirmed by probing stripped blots for β-actin. The relative density (arbitrary) of each band after normalization for β-actin is shown under each immunoblot as a fold-change compared with non–UVB-exposed control, which has been assigned an arbitrary unit 1. Significant inhibition versus UVB-exposed alone: †, $P < 0.01$; ‡, $P < 0.001$.

chronic exposure of mice to UVB markedly enhanced the expression of PCNA, cyclin D1, iNOS, and COX-2 proteins compared with control non–UVB-exposed mice. The administration of dietary GSPs at the levels of 0.2% and 0.5% (w/w) resulted in marked inhibition of UVB-induced expression of PCNA, cyclin D1, iNOS, and COX-2 proteins, which is indicated by the intensity of bands shown under each immunoblot in terms of fold-change compared with controls.

Discussion

The role of dietary botanical supplements in reduction of skin cancer has recently received widespread attention (26). It has been suggested that dietary intake of botanicals possessing substantial antioxidant properties could be a useful strategy to reduce the incidence of nonmelanoma skin cancer. We have shown previously that dietary GSPs inhibit UV-induced skin carcinogenesis in SKH-1 hairless mice in terms of tumor incidence, tumor multiplicity, and tumor size (15). The present study was designed to define the chemopreventive mechanism of action of GSPs against photocarcinogenesis. For this purpose, we used the SKH-1 hairless mouse model, a recognized model in the field of analysis of cutaneous photodamage and photocarcinogenesis. We found that administration of GSPs as 0.2% or 0.5% (w/w) supplements to a control diet resulted in significant inhibition of acute or chronic UV exposure–induced depletion of cutaneous endogenous antioxidant defense system, including GPx, catalase. and GSH. The prevention of UVB-induced depletion of antioxidant defense system will result in suppression of oxidative stress and oxidative stress–mediated adverse effects in the skin. Oxidative stress may cause damage at the cellular level, as well as at molecular level, which may result in cutaneous inflammation, lipid and protein oxidation, DNA damage, and activation or inactivation of certain enzymes (5, 7, 27, 28). Therefore, the prevention of UVB-induced depletion of antioxidant defense enzymes by dietary GSPs would provide protection against UVB-induced photodamage of the skin.

UVB-induced oxidation of lipids and proteins were used as markers of oxidative stress in the UV-exposed skin, and the oxidation of lipids and proteins in UV-exposed skin were significantly inhibited by dietary GSPs. The formation of lipid peroxides in biological membrane is a free radical–mediated event and is regulated by the availability of substrates in the form of polyunsaturated fatty acids, prooxidants that promote peroxidation, and antioxidant defenses such as α-tocopherol, GSH, β-carotene, and superoxide dismutase (28). Enhanced levels of lipid peroxides have been linked to the loss of fluidity, inactivation of membrane enzymes, and enhanced permeability to ions, which may lead to disruption of the cellular membrane (23, 28). Thus, inhibition of UV-induced lipid

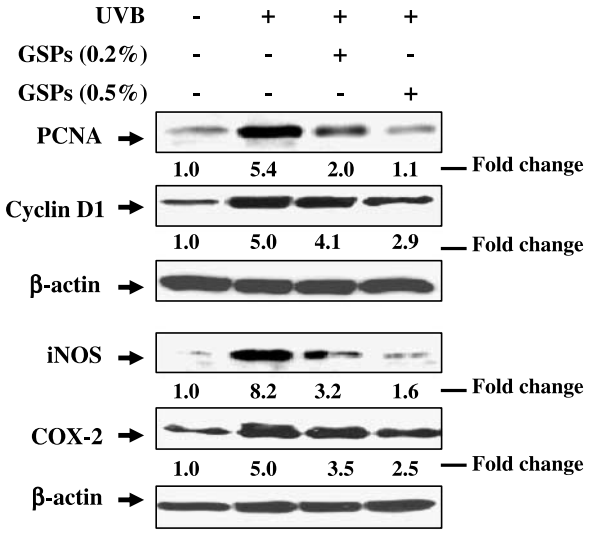

Figure 6. Dietary GSPs inhibit chronic UVB-induced expression of NF-κB–targeted genes, such as *PCNA, cyclin D1, iNOS*, and *COX-2* in mouse skin. Mice were treated as described in Fig. 1. Epidermal lysates were used to determine the levels of these proteins using Western blot analysis. A representative blot is shown from three independent experiments with almost identical observations ($n = 5$). Equivalent protein loading was confirmed by probing stripped blots for β-actin. The relative density (arbitrary) of each band after normalization for β-actin is shown under each immunoblot as a fold-change compared with non–UVB-exposed control, which has been assigned an arbitrary unit 1 in each case.

peroxidation by GSPs in an *in vivo* system should contribute to a reduction in the risk factors associated with the oxidative stress–mediated tumor-promoting effects of UV radiation. As UV-induced oxidation of protein is considered to be a contributing factor in skin disorders, such as photo-oxidative damage, the inhibition of UVB-induced protein oxidation by dietary GSPs may ameliorate photo-oxidative damage of the skin.

Dietary GSPs also inhibit UVB-induced oxidative stress in the form of total ROS and H_2O_2 generation by epidermal cells, which have been implicated in activation of various cell signaling pathways. Exposure of skin to UV radiation promotes tumor development by activating various intracellular signaling cascades that play major role in cell growth, differentiation, and proliferation, and lead to clonal expansion of UV-initiated cells into skin tumors (29). MAPKs are important upstream regulators of transcription factor activities that control cellular proliferation, differentiation, and apoptosis in response to external signals or stimuli (30). Our data show that UVB-induced phosphorylation of proteins of the MAPK family, such as ERK1/2, JNK, and p38, in mouse skin in the *in vivo* model were prevented by the dietary GSPs. ERK1/2 has been shown to be activated by tumor promoters, growth factors, and UV radiation, and to play a critical role in transmitting signals initiated by them (31). It has been shown that a potent inhibitor of the ERK pathway, BAY 43-9006, exhibits a broad spectrum of antitumor activities in breast, colon, and non–small-cell lung cancer xenograft models (32). In addition to ERK1/2, dietary GSPs inhibited UVB-induced phosphorylation of JNK and p38 proteins, and this finding was confirmed by immunohistochemical analysis on a per-cell basis. JNK regulates activator protein-1 transcription in response to environmental stress, such as UV exposure (33). Increased activator protein-1 activity has been implicated in the promotion and progression of various types of cancers, and has been involved in the stimulation of inflammation, invasion, metastasis, and angiogenesis (9, 10, 33). Therefore, inhibition of JNK activation may be a relevant molecular target for potential chemopreventive agents. It is well known that UVB induces H_2O_2 production in the target cells/tissues, which, in turn, initiates phosphorylation of MAPK and activation of downstream signals and expression of genes having direct relevance to the process of carcinogenesis (11). Therefore, dietary GSPs may prevent UVB-induced phosphorylation of MAPK proteins by preventing UVB-induced oxidative stress. Inhibition of the UVB-induced phosphorylation of MAPK proteins by GSPs may, in turn, inhibit the downstream events, such as activation of activator protein-1 and NF-κB that can contribute to photocarcinogenesis. The ERK and p38 proteins of MAPK family have been shown to modulate NF-κB activation (34). We suggest that significant inhibition of UVB-induced phosphorylation of MAPKs by GSPs might be responsible for their inhibitory effects on the activation of transcription factor NF-κB. Therefore, the MAPK and NF-κB signaling pathways are the potential targets of GSPs.

We have also observed that the enhanced phosphorylation of MAPK proteins in the UVB-exposed mouse skin is associated with the inhibition or suppression of MAPK phosphatases. It has been shown that oxidative stress or ROS promotes the phosphorylation of MAPK proteins by inhibiting MAPK phosphatases (35). These phosphatases can dephosphorylate both phosphorylated threonine and phosphorylated tyrosine (dual specificity) residues and inactivate MAPK signaling (36). MKP-1 (a family of dual-specificity protein phosphatases) has been shown to inactivate all three major MAPK proteins (ERK, JNK, and p38; ref. 37). Therefore, it is possible that UVB-induced phosphorylation of MAPK proteins in the mouse skin is inhibited by dietary GSPs through activation or restoration of MAPK phosphatases via reduction in UV-induced oxidative stress. Alternatively, MAPK may also be phosphorylated by their upstream regulators in response to UV-induced oxidative stress, and therefore requires further studies to verify these observations and to explore other mechanistic pathways.

The activation of NF-κB plays an important role in inflammation, cell proliferation, and oncogenic processes (38, 39). Indeed, the signaling pathways leading to the regulation of NF-κB activity have been targeted in the development of chemopreventive agents. The activation of NF-κB by extracellular inducers depends on the phosphorylation and subsequent degradation of IκB proteins. Activation of NF-κB is achieved through the action of a family of serine/threonine kinases known as IKK. The IKK (IKKα and/or IKKβ) phosphorylate IκB proteins and the members of the NF-κB family. Therefore, the inhibitors of IKK have been considered as specific regulators of NF-κB. NF-κB is commonly activated by oxidants, including H_2O_2 (40) and by agents that generate ROS, including UV radiation (41, 42). In our current *in vivo* study, we observed that NF-κB/p65 is activated after UVB exposure and subsequently translocated to the nucleus; however, its activation and translocation to the nucleus was effectively inhibited by dietary GSPs. UVB irradiation also resulted in an increased degradation of IκBα protein. As GSPs block IκBα degradation in UVB-exposed skin, our study suggests that the inhibitory effect of GSPs on UV-induced NF-κB/p65 activation may be mediated through the inhibition of proteolysis of the IκBα protein. It is documented that IκBα is bound to NF-κB/p65 through a protein-protein interaction, and thus this interaction prevents migration of NF-κB/p65 into the nucleus (39). Our data suggest that treatment of mice with dietary GSPs inhibits UVB-induced activation and nuclear translocation of NF-κB/p65 through the inhibition of activation of IKKα and degradation of IκBα proteins.

There is evidence that NF-κB regulates a wide variety of genes that encode proteins that are involved in inflammation and carcinogenesis (43–45). Activation of NF-κB can up-regulate the expression of proinflammatory cytokines and inflammatory gene products, such as COX-2 and iNOS (44, 45). It also is regarded as a key regulator of cell proliferation through its direct or indirect effects on

cell cycle regulation, including its effects on cyclin D1, a cyclin that is expressed early in the cell cycle and is important for DNA synthesis (46). Most of these genes have been shown to be up-regulated in human cancers, suggesting that inhibition of NF-κB and subsequently NF-κB–targeted genes might inhibit the development of cancers, including skin cancer. In the present study, we observed that chronic exposure of mouse skin to UVB enhanced the levels of NF-κB–responsive proteins, such as PCNA, cyclin D1, iNOS, and COX-2. Importantly, the expressions of these proteins in mouse skin were inhibited markedly by dietary GSPs in this *in vivo* model. The inhibition of expression of these NF-κB–targeted genes in UV-exposed mice may explain the antiproliferative and antioxidative effects of GSPs. It is important to mention that the inhibition of the expression of PCNA, cyclin D1, COX-2, or iNOS in UVB-exposed skin by dietary GSPs may be independent to the inhibition of NF-κB, and therefore requires further studies. Because of the role of NF-κB pathway in cell survival and proliferation, our findings regarding the effects of GSPs on this pathway suggest that it represents a key molecular target of GSPs. Inhibition of NF-κB activation has been shown to be an important mechanism of action of some other botanicals and/or polyphenols, including green tea polyphenols (47), resveratrol (48), and silibinin (49). It can be speculated that a combination of these other botanicals with GSPs, either in the diet or topically, might improve their chemopreventive or chemotherapeutic potential against photocarcinogenesis. However, mechanism-based *in vivo* studies are required to validate this concept. Moreover, GSPs and other chemopreventive agents, such as green tea polyphenols, are potent antioxidant in nature and therefore have shown almost similar chemopreventive mechanism of action against UVB radiation–induced adverse biological effects in the skin. These chemopreventive agents have shown the beneficial effects against UV radiation whether applied topically or given in drinking water/diet (26).

This *in vivo* study provides conclusive evidence that dietary GSPs have the potential to attenuate UVB-induced oxidative stress and to inhibit the activation of the cellular signaling cascades involving the MAPK and NF-κB pathways that are associated with high risk of photocarcinogenesis. Based on the current observations, it seems that photoprotective effects of GSPs are mediated, at least, through (*a*) prevention of UVB-induced depletion of endogenous antioxidant defense system, (*b*) prevention of photo-oxidative damage of lipids and proteins, and (*c*) inhibition of activation of MAPK and NF-κB signaling pathways, which seems to be mediated through the reactivation or restoration of MAPK phosphatases in the skin. Because the early activation of cellular signaling pathways in response to UV irradiation is involved in UVB-induced inflammatory reactions, photoaging, and photocarcinogenesis, the use of GSPs as a dietary supplement may have beneficial effects in protecting against cutaneous disorders in UV-exposed skin.

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