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Treatment of green tea polyphenols in hydrophilic cream prevents UVB-induced oxidation of lipids and proteins, depletion of antioxidant enzymes and phosphorylation of MAPK proteins in SKH-1 hairless mouse skin

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The use of botanical supplements has received immense interest in recent years to protect human skin from adverse biological effects of solar ultraviolet (UV) radiation. The polyphenols from green tea are one of them and have been shown to prevent photocarcinogenesis in animal models but their mechanism of photoprotection is not well understood. To determine the mechanism of photoprotection in *in vivo* mouse model, topical treatment of polyphenols from green tea (GTP) or its most chemopreventive constituent (−)-epigallocatechin-3-gallate (EGCG) (1 mg/cm^2 skin area) in hydrophilic ointment USP before single (180 mJ/cm^2) or multiple UVB exposures (180 mJ/cm^2, daily for 10 days) resulted in significant prevention of UVB-induced depletion of antioxidant enzymes such as glutathione peroxidase (78–100%, $P < 0.005$–0.001), catalase (51–92%, $P < 0.001$) and glutathione level (87–100%, $P < 0.005$). Treatment of EGCG or GTP also inhibited UVB-induced oxidative stress when measured in terms of lipid peroxidation (76–95%, $P < 0.001$), and protein oxidation (67–75%, $P > 0.001$). Further, to delineate the inhibition of UVB-induced oxidative stress with cell signaling pathways, treatment of EGCG to mouse skin resulted in marked inhibition of a single UVB irradiation-induced phosphorylation of ERK1/2 (16–95%), JNK (46–100%) and p38 (100%) proteins of MAPK family in a time-dependent manner. Identical photoprotective effects of EGCG or GTP were also observed against multiple UVB irradiation-induced phosphorylation of the proteins of MAPK family *in vivo* mouse skin. Photoprotective efficacy of GTP given in drinking water (d.w.) (0.2%, w/v) was also determined and compared with that of topical treatment of EGCG and GTP. Treatment of GTP in d.w. also significantly prevented single or multiple UVB irradiation-induced depletion of antioxidant enzymes (44–61%, $P < 0.01$–0.001), oxidative stress (33–71%, $P < 0.01$) and phosphorylation of ERK1/2, JNK and p38 proteins of MAPK family but the photoprotective efficacy was comparatively less than that of topical treatments of EGCG and GTP. Lesser photoprotective efficacy of GTP in d.w. in comparison with topical application may be due to its less bioavailability in skin target cells. Together, for the first time a cream based formulation of green tea polyphenols was tested in this study to explore the possibility of its use for the humans, and the data obtained from this *in vivo* study further suggest that GTP could be useful in attenuation of solar UVB light-induced oxidative stress-mediated and MAPK-caused skin disorders in humans.

Abbreviations: AP-1, activator protein-1; DNPH, dinitrophenylhydrazine; d.w., drinking water; EGCG, epigallocatechin-3-gallate; ERK, extracellular signal regulated kinase; GSH, glutathione; GPx, glutathione peroxidase; GTP, green tea polyphenols; LPO, lipid peroxidation; MAPK, mitogen activated protein kinases; JNK, c-jun N-terminal kinase; ROS, reactive oxygen species; UV, ultraviolet.

Introduction

Epidemiological, clinical and biological studies have implicated that solar ultraviolet (UV) light, especially UVB (290–320 nm) wavelength, is a complete carcinogen and repeated exposures can lead to the development of melanoma and non-melanoma skin cancer (1–6). Melanoma and non-melanoma skin cancers are by far the most common type of malignancy and has a tremendous impact on public health and healthcare expenditures. The incidence of skin cancer is continuing to grow at an alarming rate, and more than 1 million new cases of basal cell carcinomas and squamous cell carcinomas are diagnosed each year in USA and this increase is expected to continue as the population ages and larger amounts of UV radiation reaches the Earth's surface because of depletion of the ozone layer (3–7). Additionally, the increased tendency of individuals to obtain a rapid tan and the use of tanning booths are also implicated in the high risk of melanoma and non-melanoma skin cancers (1–7). It has also been suggested that melanoma is considerably more life threatening than the other skin cancers (1,3,7). The detrimental effects of UV irradiation have been attributed to its ability to induce inflammatory responses (8,9), and oxidative stress that damage vital macromolecules like proteins, lipids and DNA which have been shown to be implicated in the onset of several skin diseases including skin cancer (10–13).

There is considerable evidence that UV-induced oxidative stress mediates the phosphorylation or activation of protein kinases through a series of cascades such as mitogen activated protein kinases (MAPK) (14–18). Three structurally related but biochemically and functionally distinct MAPK signal transduction pathways have been identified in mammalian cells such as extracellular signal regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK) and p38. These MAPK are mediators of signal transduction from the cell surface to the nucleus and play a major role in triggering and coordinating gene responses (19). Whereas ERKs are predominantly activated by mitogenic signals, JNK and p38 are primarily activated by environmental stresses such as UV radiation, inflammatory cytokines, heat shock and DNA-damaging agents (16,19,20). Transient activation of ERK is responsible for proliferation and differentiation (21), and has also been shown to be involved in the tumor promotion process especially stimulated by the oxidant state (22). Phosphorylation of

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JNK and p38 can mediate differentiation, inflammatory responses and cell death (21,23,24). There are evidences showing that antioxidants can attenuate MAPK activation (22,25), thereby suggesting that MAPK signaling cascades are important targets of reactive oxygen species (ROS).

Green tea (*Camellia sinensis*) is consumed as a popular beverage worldwide. Epidemiological as well as experimental studies conducted in laboratory animals have detected an association between tea consumption and decreased cancer risk (26,27). In recent years, cancer chemoprevention by biologically active dietary or non-dietary botanical supplements with substantial antioxidant activity has generated immense interest in view of their putative role in attenuating the risk of cancer incidence. Against this background, green tea polyphenols (GTP) are promising chemoprotective agents against several major epithelial and non-epithelial cancers (26). The major polyphenols or epicatechin derivatives present in green tea are (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin-3-gallate (EGCG). Of these components, EGCG has been shown to be the most potent antioxidant and major chemopreventive constituent of green tea and has been the focus of a great deal of attention in cancer prevention studies (reviewed in refs 26–28). Earlier studies have demonstrated that oral feeding of green tea extract or GTP (a mixture of four major epicatechin derivatives) in drinking water (d.w.) or topical application of GTP or EGCG in organic solvents, like acetone, to the mouse skin inhibited 7,12-dimethylbenz[*a*]anthracene-initiated and 12-*O*-tetradecanoylphorbol-13-acetate-promoted (29) and UV-induced adverse biological effects associated with skin carcinogenesis (27,30–34). In efforts to demonstrate the anticarcinogenic mechanism of action of GTP or EGCG against UVB irradiation, we showed that topical application of GTP in acetone afforded protection against single UVB irradiation-induced depletion of glutathione reductase, infiltration of inflammatory leukocytes, and nitric oxide and H_2O_2 production in mouse skin (30,35) and human skin as well (36). Various mechanisms have been proposed for the anticarcinogenic effects of green tea, including the modulation of signal transduction pathways that leads to the inhibition of cellular proliferation, induction of apoptosis of pre-neoplastic and neoplastic cells but mainly confined in *in vitro* systems (27,28,37). Although the UV-induced MAPK signaling pathways have been the subjects of intense interest, much of the studies are restricted to *in vitro* cell culture system. Moreover, very importantly, in all the previous *in vivo* studies organic solvents like acetone were used as a vehicle for the topical application of GTP or EGCG (reviewed in refs 26–28). Use of organic solvents in skin care products or in topical treatment of pharmacologic agents for human use does not seem to be clinically appropriate. Dvorakova *et al.* (38) have reported that topical treatment of EGCG with hydrophilic cream/ointment USP achieved high concentration in the skin. Therefore, for the first time, to determine the efficacy of GTP we selected to use this hydrophilic ointment USP as a suitable cream based vehicle that could be employed for the beneficial effects of GTP in humans. The present study was designed and further extended in *in vivo* SKH-1 hairless mouse skin model to evaluate the photoprotective effects of purified GTP (contain 86% epicatechin derivatives) or EGCG (>98% pure) in hydrophilic cream (10%, w/w). Simultaneously, we were also interested to determine the photoprotective effects of GTP given to mice in d.w. (0.2%, w/v) and to compare the effects with topical treatments. The photoprotective effects of GTP or EGCG were determined against single as well as multiple UV exposures-induced: (i) depletion of endogenous antioxidant defence enzymes, such as glutathione peroxidase (GPx), catalase and glutathione (GSH), (ii) markers of oxidative stress such as lipid peroxidation (LPO) and protein carbonyls formation and (iii) phosphorylation of MAPK proteins *in vivo* mouse model by using analytical assays and western blot analysis.

Materials and methods

Animals

Female SKH-1 hairless mice of 6–8 weeks old were purchased from Charles River Laboratory (Wilmington, MA) and were housed in our animal facility. Mice were kept in groups of four per cage and fed with Teklad chow diet and water *ad libitum*. The animals were acclimatized for ~1 week before use and maintained throughout at standard conditions: 24 ± 2°C temperature, 50 ± 10% relative humidity and 12 h light/12 h dark cycle. The animal protocol for this study was approved by Institutional Animal Care and Use Committee of the University of Alabama at Birmingham, Birmingham, Alabama, in accordance with the current US Department of Agriculture, Department of Health and Human Services regulations and standards.

Antibodies and reagents

Primary antibodies to phosphorylated and non-phosphorylated ERK1/2, SAPK/JNK and p38 were purchased from Cell Signaling Technology (Beverly, MA). OxyBlot™ Protein Oxidation Detection Kit was purchased from INTERGEN Company (Purchase, NY). Purified EGCG (>98% pure) and GTP (containing 86% catechins) were obtained as a gift from Dr Yukihiko Hara (Mitsui Norin, Shizuoka, Japan). Purified GTP used in this study has the following composition: EC = 10.4%, EGC = 8.3%, EGCG = 55.8%, gallocatechin gallate = 4.4% and ECG = 6.9%. In all the experiments conducted in this study, EGCG or GTP was uniformly mixed in hydrophilic ointment USP (Fougera & Co., Melville, NY) and was topically applied (≈1 mg/cm^2 skin area) to the mouse skin 20–25 min before UVB exposure. This hydrophilic ointment or cream is prescribed for external use as an ointment or cosmetic base. All other chemicals were purchased from authentic sources and of highest grade and purity.

UV irradiation

UVB irradiation was performed as described earlier (35). Briefly, dorsal skin was exposed to UV irradiation from a band of four FS-20 fluorescent lamps from which short wavelengths of UVB (280–290 nm) and UVC normally not present in natural solar light were filtered out using Kodacel cellulose film (Eastman Kodak, Rochester, NY). The majority of the resulting wavelengths after Kodacel filtration were in UVB (290–320 nm) and UVA range with peak emission at 314 nm as monitored. The UVB emission was monitored before each exposure of the mice with an IL-1700 phototherapy radiometer equipped with an IL SED 240 detector fitted with a W side angle quartz diffuser and a SC5 280 filter (all from International Light, Newburyport, MA). During UVB irradiation mice were held in dividers separated by Plexiglas. Mice were exposed to either a single UVB dose of 180 mJ/cm^2 or multiple UVB exposures (180 mJ/cm^2/day) for 10 consecutive days.

Use of hydrophilic ointment as a vehicle for the topical treatment of EGCG or GTP

For the first time, we tested the efficacy of GTP in hydrophilic ointment or cream based formulation against UV-induced adverse biological effects in the mouse skin. Topical treatment of EGCG or GTP was given at the dose of ≈1 mg/cm^2 skin area in formulation. Current dose of EGCG or GTP was selected based upon our prior studies where the treatment of this dose resulted in significant protection against photodamage (27,35,36). Simultaneously, another group of mice was given GTP (0.2%, w/v) in d.w. to determine the photoprotective effect on UV-induced adverse biological effects similar to topical treatment. GTP in d.w. was given for 10 days before and during UVB exposure protocol. The dose of GTP (0.2%, w/v) in d.w. was also selected based on our prior chemopreventive observations (30). Mice of control (non-UV) as well as UV alone exposed groups were also maintained which were either topically treated with the same amount of hydrophilic cream alone or d.w. without GTP treatment.

Collection of skin biopsies and preparation of cytosols and microsomal fractions

In the case of a single UV exposure, mice were killed at desired time points after UV exposure and skin biopsies were obtained. In the case of multiple UV

exposures, mice were killed 24 h after the last UV exposure and skin biopsies were obtained. For enzymic analysis and markers of oxidative stress, epidermal cytosol and microsomal fractions were prepared as described earlier (36). Briefly, epidermal layer was homogenized with a Polytron homogenizer in PBS buffer containing potassium chloride and centrifuged at 18 000 *g* for 15 min at 4°C to prepare cytosolic and microsomal fractions (36). Epidermal cytosols were used to determine the endogenous antioxidant defence parameters like GSH, GPx and catalase whereas microsomal fraction was used to determine LPO.

Assays for endogenous antioxidant enzymes

The levels of endogenous antioxidant defence enzymes such as GPx (EC 1.11.1.9), catalase (EC 1.11.1.6) and GSH were measured in epidermal cytosolic fractions following the methods of Flohe and Gunzler (39), Nelson and Kiesow (40) and Akerboom and Sies (41), respectively.

Assay for LPO

LPO was determined in epidermal microsomal fractions from the different treatment groups using thiobarbituric acid (TBA) reaction method (42). Briefly, the reaction was carried out using 0.2 ml of the epidermal microsomes and is treated with 0.2 ml of 8.1% SDS and 3 ml of TBA reagent (equal volumes of 0.8% TBA and 20% acetic acid, pH 3.5). Total volume was made up to 4 ml with distilled water and kept at 95°C for 1 h in a water bath. Color was extracted with *n*-butanol and pyridine (15:1 v/v). The absorbance was measured at 530 nm. The values are obtained from the standard graph generated from 1,1,3,3- tetraethoxy propane. The curve was linear up to 20 nM/ml MDA.

Assay for protein carbonyls

The amount of protein carbonyls was measured as a marker of protein oxidation following the method of Cao and Cutler using dinitrophenylhydrazine (DNPH) (43). Briefly, the DNA from the skin lysates was removed by adding streptomycin sulfate to a final concentration of 1%. The samples were then dialyzed in 12 000–14 000 molecular weight cut off membrane bags (Spectrum Laboratories, Rancho Dominguez, CA) against water at 4°C for 2.5 h. The precipitate was centrifuged at 10 000 *g* for 10 min. The supernatant was used for the carbonyl assay. The supernatant (containing ~1–1.5 mg protein/ml) was treated with 4 ml of 12.5 mM DNPH in 2.5 M HCl and incubated at room temperature for 1 h. Protein was precipitated with a final concentration of 10% trichloroacetic acid. The pellets were washed vigorously after breaking the pellet by a glass rod with 4 ml of ethanol:ethyl acetate (1:1, v/v) until no DNPH was extracted into the solvent. The pellets were dissolved in 6 M guanidine hydrochloride at 37°C for 20 min. Insoluble materials were removed by centrifugation and absorbance was measured at 370 nm. Negative control was also maintained by removing proteins through its precipitation with trichloroacetic acid before adding DNPH solution. The protein concentration was determined from the blank by obtaining the absorbance at 287 nm. The reactive protein carbonyl content was calculated from the molar extinction coefficient 22 000 M^{-1} cm^{-1}.

Western blotting for protein oxidation

Western blotting to detect oxidized proteins was performed using OxyBlot™ Protein Oxidation Detection kit (Intergen Company, Purchase, NY) following the manufacturer's protocol. Briefly, samples of 10 μg proteins were subjected to DNPH derivatization. Incubation of equal aliquots with a control solution lacking DNPH served as negative control. The dinitrophenylhydrazone-derivatized protein samples were separated by 10% SDS–PAGE gel electrophoresis, and blotted onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin in TBS-Tween and incubated with a rabbit anti-dinitrophenylhydrazone antibody in 1% bovine serum albumin in TBS-Tween (1:150) for 1 h at room temperature and then with a peroxidase-coupled goat antirabbit IgG antibody for 1 h at room temperature. The membranes were then treated with chemiluminescence to visualize protein bands using ECL detection system (Amersham Life Sciences, Arlington, IL). Protein carbonylation was determined by autoradiography with XAR-5 films.

Preparation of skin lysates and immunoblotting of MAPK proteins

Epidermal layer was separated as described earlier (44) and transferred immediately to a tube containing ice-cold cell lysis buffer and protease inhibitors (Tris–HCl: 50 mM, pH 7.4; 1% NP-40; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mM sodium orthovanadate; 1 mM NaF and aprotinin and leupeptin 1 μg/ml each) (37). The epidermal tissue was homogenized using Polytron homogenizer, centrifuged at 14 000 r.p.m. for 15 min at 4°C and the supernatants were collected for western blot analysis. Supernatants either used immediately or stored at −70°C. The protein content was determined in the supernatant by the DC BioRad protein assay kit using the manufacturer's protocol.

To determine the level of phospho-specific MAPK proteins in different treatment groups, proteins were immunoprecipitated following the procedure of Ahmad *et al*. (37) and thereafter subjected to western blotting. Briefly, lysates (200 μg protein/sample) were diluted to 1 ml with lysis buffer, and pre-cleared with 20 μl protein A/G–agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) by gentle rotation at 4°C for 1 h followed by the removal of beads by centrifugation for 5 min at 2000 r.p.m.. The pre-cleared lysates were incubated overnight at 4°C with continuous rotation with 2 μg primary antibody and 20 μl protein A/G–agarose beads. Thereafter, immunocomplexes were collected by centrifugation at 2000 r.p.m. for 5 min, and washed four times with lysis buffer. The sample buffer containing samples (50 μg protein) were resolved over 10% polyacrylamide–SDS gels and transferred to a nitrocellulose membrane. The membrane was blocked with blocking buffer [5% non-fat dry milk in 1% Tween 20 in 20 mM Tris-buffered saline (pH 7.5)] by incubating it for 1 h at room temperature followed by incubation with the appropriate primary antibody as recommended by the manufacturer in blocking buffer containing 5% BSA or non-fat dry milk overnight at 4°C. This was followed by incubation with the appropriate secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technology, Beverly, MA). Protein bands were visualized by chemiluminescence using ECL detection system (Amersham Life Sciences, Arlington Heights, IL) and autoradiography with XAR-5 film (Eastman Kodak, Rochester, NY). To compare relative intensity of each protein band from different treatment groups in western blot, computerized densitometry was performed using OPTIMAS 6.1 software program.

Statistical analysis

All experiments were performed at least three times with each assay conducted in duplicate or triplicate. The results are expressed as means ± SD in terms of percent of control in case of antioxidant enzymes and markers of oxidative stress. Statistical analysis of all data between UV exposure alone and EGCG/GTP treatment +UV exposure groups were analyzed by Student's *t*-test. A P value <0.05 was considered statistically significant.

Results

Initially, we performed preliminary experiments to test the stability of EGCG or GTP in hydrophilic ointment (10%, w/w) under different storage temperatures, like at 4 and 25°C (room temperature). Change in color of the EGCG or GTP formulations was the main criteria to test the stability of these formulations. These observations indicated that the formulations containing either EGCG or GTP are stable at least 6–7 months at 4°C while remain stable for ~3–4 months at 25°C.

Treatment of green tea polyphenols prevents UVB-induced depletion of GPx, catalase and GSH in the skin

It is established that UV exposure depletes the antioxidant defence capability of the skin at UV-irradiated site (7,36). Our data demonstrated that UV irradiation to the skin, either single or multiple exposures, resulted in significant depletion of the GPx, respectively, by 31 and 44% in comparison with control (non-UV exposed) animals (Figure 1A). However, the treatment of EGCG or GTP to animals afforded almost 100% protection against single UV exposure-induced depletion of GPx activity. In animals exposed to multiple UVB doses, treatment of EGCG or GTP resulted in 78–80% ($P < 0.005$) protection of UV-induced depletion of GPx activity as shown in Figure 1A. Treatment of GTP in d.w. also resulted in prevention of single or multiple UV irradiation-induced depletion of GPx activity in the skin by 55–61% ($P < 0.01$). The data indicated that photoprotective effects of topical treatment of EGCG and GTP were almost similar, as shown in Figure 1A.

Catalase is another endogenous antioxidant enzyme involved in the catalytic conversion of H_2O_2 to oxygen and water and thus reduces the level of oxidative stress. Single and multiple exposures of UVB to skin markedly reduced catalase activity, respectively, by 54 and 85% as shown in Figure 1B. The topical treatments with EGCG and GTP in cream significantly prevented single and multiple UVB exposure-induced depletion of catalase activity, respectively, by 60 and 51% ($P < 0.001$), and 90 and 92% ($P < 0.001$), as shown in

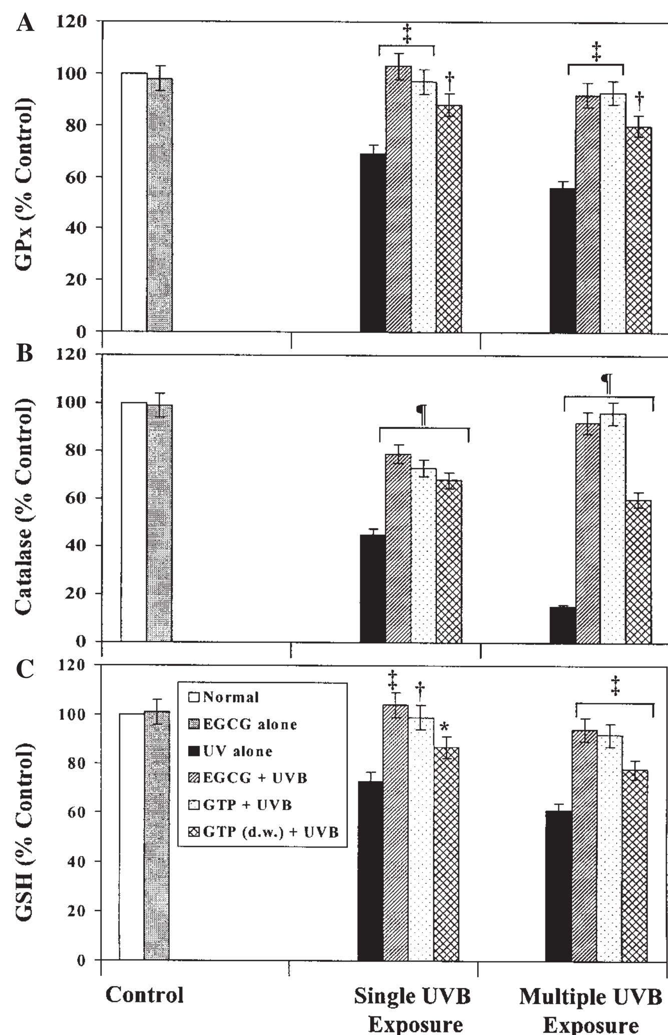

Fig. 1. Treatment of green tea polyphenols prevents single as well as multiple UVB exposure-induced depletion of GPx, catalase and GSH in SKH-1 hairless mouse skin. Mice were irradiated with either a single exposure (180 mJ/cm^2) or multiple exposures of UVB (180 mJ/cm^2 each day for 10 consecutive days). EGCG or GTP was topically applied in hydrophilic ointment USP (≈1 mg/cm^2 skin area). GTP treatment was also given in d.w. (0.2%, w/v) 10 days prior to UVB exposure and during UVB exposure protocol. Animals were killed at 24 h after the last UVB exposure and skin biopsies were collected and cytosols were prepared as described in the Materials and methods section. Skin biopsies were pooled from three mice in different treatment groups to prepare cytosolic fraction, and experiments were repeated three times to analyze antioxidant defence enzymes. Data are presented as means ± SD in terms of percent of control. Treatments were as shown in (C). (**A**) Topical treatment of EGCG or GTP and GTP in d.w. to mice prevents single and multiple UVB exposure-induced depletion of GPx activity. (**B**) Topical treatment of EGCG or GTP, and GTP in d.w. to mice prevents single and multiple UVB exposure-induced depletion of catalase activity. (**C**) Topical treatment of EGCG or GTP, and GTP in d.w. to mice prevents single and multiple UVB exposure-induced depletion of GSH. *Significant prevention in depletion of antioxidant enzymes by EGCG or GTP + UVB treatment versus UVB irradiation alone, $P < 0.05$. †Significant prevention in depletion of antioxidant enzymes by EGCG or GTP + UVB treatment versus UVB irradiation alone, $P < 0.01$. ‡Significant prevention in depletion of antioxidant enzymes by EGCG or GTP + UVB treatment versus UVB irradiation alone, $P < 0.005$. ¶Significant prevention in depletion of antioxidant enzymes by EGCG or GTP + UVB treatment versus UVB irradiation alone, $P < 0.001$.

Figure 1B. When GTP was administered in d.w. to UVB irradiated mice, 44 and 53% ($P < 0.001$) prevention was observed, respectively, against single and multiple UVB exposure-induced depletion of catalase activity.

GSH is an important endogenous antioxidant whose nucleophilic and reducing properties play a central role in metabolic pathways, as well as in the antioxidant system of aerobic cells. UVB irradiation of the mouse skin resulted in reduction of the GSH level after single exposure (27%), as well as after multiple exposures (39%) compared with control (non-UVB exposed) mice as shown in Figure 1C. Topical application of EGCG or GTP on the mouse skin completely prevented a single UVB irradiation-induced depletion of GSH. In case of multiple UVB exposures, treatment of EGCG or GTP resulted in >87% ($P < 0.005$) prevention of UVB-induced depletion of GSH. Similarly, administration of GTP given in d.w. also produced preventive effects against single (55%, $P < 0.05$) and multiple (48%, $P < 0.05$) UVB irradiation-induced depletion of GSH level as shown in Figure 1C.

Treatment of green tea polyphenols prevents UVB-induced oxidation of lipids and proteins in the skin

One of the hallmarks of oxidative stress is the formation of oxidized macromolecules such as lipid peroxides or LPO. Therefore, we used LPO as the marker of photooxidative damage. In the present study, we found that both single and multiple exposures of UVB to mouse skin increased lipid peroxidation (Figure 2A). When mice were exposed to a single UV exposure of 180 mJ/cm^2, the level of lipid peroxidation was increased to 180% of control (non-UV exposed) mice. Similarly, multiple exposures of UVB to mouse skin significantly increased LPO (260%, $P < 0.001$) compared with non-UV irradiated mice. Treatment of EGCG or GTP resulted in significant prevention of UV-induced LPO against single (>95%, $P < 0.0001$) and multiple UVB exposures (76%, $P < 0.001$). Administration of GTP in d.w. also resulted in significant prevention of UV-induced LPO by 71 ($P < 0.001$) and 34% ($P < 0.05$), respectively, against a single and multiple exposures of UV radiation.

Oxidation of some amino acid residues of proteins such as lysine, arginine and proline leads to the formation of carbonyl derivatives that affects the nature and function of proteins (45). The presence of carbonyl groups has become a widely accepted measure of oxidative damage of proteins under conditions of oxidative stress, which react with DNPH to form stable hydrazone derivatives (46). The level of carbonyls in normal healthy human skin was found to be low, however, the level increases extensively after UV exposure (47). Therefore, we analyzed UV-induced protein carbonyl formation as a measure of oxidative stress in the mouse skin. We found that single and multiple UV exposures of the skin resulted, respectively, in a 10- and 21-fold increase in the level of protein carbonyls in comparison with non-UV exposed animals when measured at 24 h after UV exposure as shown in Figure 2B. Topical treatments of EGCG and GTP significantly inhibited single UVB irradiation-induced protein carbonyls formation, respectively, by 72 and 67% ($P < 0.001$). Similarly, treatment of EGCG and GTP also significantly inhibited multiple UVB irradiation-induced protein carbonyl formation, respectively, by 75 and 70%. Treatment of GTP in d.w. also resulted in 33 and 42% inhibition of protein carbonyl formation after single and multiple UV exposures, respectively, as shown in Figure 2B. Additionally, the inhibition of a single and multiple UVB irradiation-induced protein oxidation in mouse skin by GTP was confirmed by western blot analysis, as shown in Figure 2C. UVB irradiation-induced protein oxidation is evident from the darker bands of different proteins (Figure 2C).

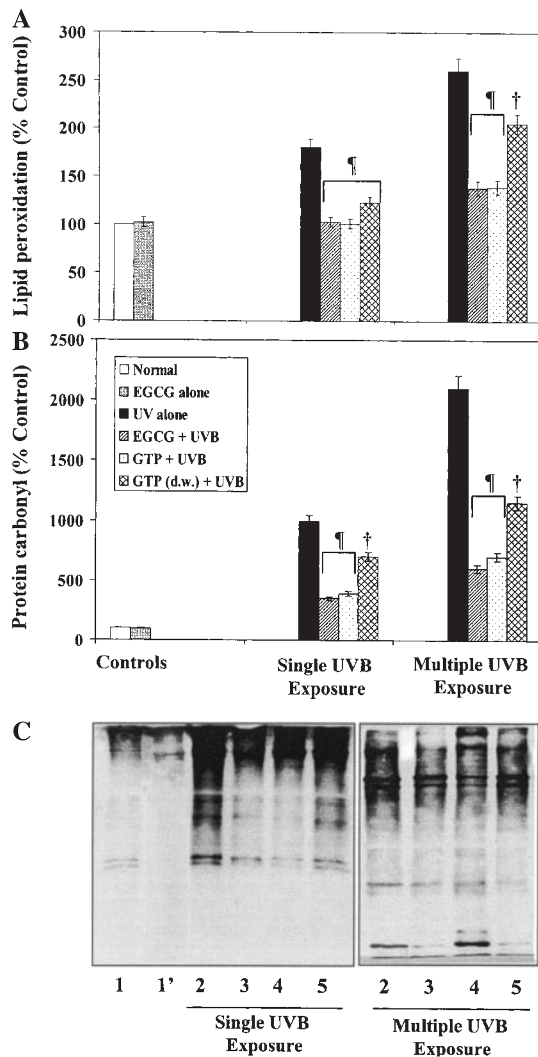

Fig. 2. Treatment of green tea polyphenols prevents single as well as multiple UVB exposure-induced markers of oxidative stress in SKH-1 hairless mouse skin. Mice were irradiated with either a single exposure (180 mJ/cm^2) or multiple exposures of UVB (180 mJ/cm^2 each day for 10 consecutive days). EGCG or GTP was topically applied in hydrophilic ointment USP ($\approx$1 mg/cm^2 skin area). GTP treatment was also given in d.w. (0.2%, w/v) 10 days prior to UVB exposure and during UVB exposure protocol. Animals were killed at 24 h after the last UVB exposure and skin biopsies were collected. Epidermal microsomal fraction was prepared for LPO, and skin lysates were prepared for the determination of protein carbonyls as described in the Materials and methods section. Skin biopsies were pooled from three mice in different treatment groups to prepare microsomes or lysates, and experiments were repeated three times. Treatments were as shown in (B). Data are presented as means $\pm$ SD in terms of percent of control. (**A**) Topical treatment of EGCG or GTP, and GTP given in d.w. to mice prevents single and multiple UVB exposure-induced epidermal LPO. (**B**) Topical treatment of EGCG or GTP, and GTP given in d.w. to mice prevents single and multiple UVB exposure-induced protein carbonyls (a marker of protein oxidation) formation. Protein carbonyls were determined employing analytical assay as described in the Materials and methods section. (**C**) Prevention of UVB-induced protein carbonyls or protein oxidation in mouse skin was determined by western blot analysis as detailed in the Materials and methods section. Treatment with EGCG or GTP to single UVB (left panel) or multiple UVB (right panel) irradiated skin resulted in marked inhibition of UVB-induced oxidation of proteins. Comparable intensity of various proteins in different treatment groups is visible by the thickness of the bands. A representative blot is shown from three separate experiments with similar results. Treatment groups are: 1 = Control (non-UV); 1′ = EGCG alone (non-UV); 2 = UVB alone; 3 = EGCG + UVB; 4 = GTP (topical) + UVB; and 5 = GTP (d.w.) + UVB. †Significant prevention of LPO by EGCG + UVB and GTP + UVB treatment versus UVB exposure alone, $P < 0.01$. ¶Significant prevention of protein oxidation by EGCG + UVB and GTP + UVB treatment versus UVB irradiation alone, $P < 0.001$.

Further, topical treatment of EGCG and GTP, and GTP in d.w. to mice resulted in inhibition of UVB-induced protein oxidation in mouse skin.

Kinetics of a single UVB irradiation-induced phosphorylation of MAPK, and prevention by EGCG treatment in in vivo mouse skin

The MAPK proteins are important upstream regulators of transcription factor activities and their signaling is critical to the transduction of a wide variety of extracellular stimuli into intracellular events (48). The MAPK family proteins like ERK1/2, JNK and p38 are thought to interplay in the control of cellular events like proliferation, differentiation and apoptosis in response to external stimuli and have been implicated in multi-stage skin carcinogenesis (49). Therefore, we were interested in examining the chemopreventive effect of EGCG, which was found to be the most photoprotective agent in present studies, on UVB-induced phosphorylation of MAPK proteins. For determining the kinetics of UVB-induced phosphorylation of MAPK proteins *in vivo* system, three early time points such as 1, 3 and 6 h, and one delayed time point, such as 48 h, after a single UV exposure was selected. Among these selected time points, UVB-induced phosphorylation of ERK1/2 was observed throughout 1–48 h after UVB exposure, but maximum phosphorylation was noted at 1 h after UVB exposure of mouse skin as shown in Figure 3A. Western blotting and subsequently densitometric analyses of relative intensity of bands indicated that topical treatment of EGCG in hydrophilic cream markedly inhibited UVB-induced phosphorylation of ERK1/2 (91–95%) at 1 h after UV exposure but comparatively less inhibition was observed at 6 (16%) and 48 h (63%) after UV exposure, as shown in Figure 3A.

In contrast to UVB-induced ERK phosphorylation, the marked induction in UVB-induced phosphorylation of JNK [JNK1 (p46) and JNK2 (p54)] was observed at 6 h after UV irradiation and reaches its basal level at 48 h (Figure 3B). Western blot and subsequently measurement of relative intensity of bands revealed that treatment of EGCG markedly inhibited UVB-induced phosphorylation of JNK1 and JNK2 at 1 (100%) and 6 h (46–53%) after UV exposure.

UVB exposure to skin induced phosphorylation of p38, and this phosphorylation was observed within 5 min after UV irradiation. The maximum UVB-induced phosphorylation of p38 was observed at 30 min after UV exposure and thereafter declined as shown in Figure 3C. Topical treatment of EGCG completely inhibited UV-induced phosphorylation of p38 at all the time points studied. It was also observed that EGCG treatment to UVB exposed skin reduced the level of p38 below the basal level at all the time points studied when compared with control (non-UVB exposed) mice. However, topical treatment of EGCG alone to mouse skin did not induce phosphorylation of either ERK1/2, JNK or p38 proteins of MAPK family (data not shown).

Treatment of green tea polyphenols prevents multiple UVB exposure-induced phosphorylation of MAPK in in vivo mouse skin

Once determined the kinetics and photoprotective effects of EGCG on UVB-induced phosphorylation of MAPK after single UVB exposure, we were further interested in determining the effect of EGCG and GTP on the phosphorylation of MAPK proteins after multiple exposures of UVB radiation in *in vivo* mouse skin. Therefore, in this experiment, the photoprotective

effects of the topical treatment of EGCG and GTP (1 mg/cm^2 skin area), as well as GTP in d.w. (0.2%, w/v), were determined. Western blot analysis revealed that ERK2 was relatively more phosphorylated than ERK1 in UV exposed samples, and the level of phosphorylation of ERK2 was several-fold in UV-exposed skin compared with non-UVB exposed skin samples, as shown in Figure 4A. Densitometry of bands indicated that topical treatment of EGCG to skin was the most potent chemopreventive agent, and resulted in >95% inhibition of UVB-induced phosphorylation of ERK1/2. Topical treatment of GTP to the skin was found less effective than EGCG, and resulted in inhibition of UVB-induced phosphorylation of ERK2 and ERK1, respectively, by 54 and 86%. Treatment of GTP in d.w. to mice also inhibited UVB-induced phosphorylation of ERK2 and ERK1 by 53 and 47%, respectively (Figure 4A). The data also clearly indicated that in addition to protection by topical treatment of EGCG and GTP, treatment of GTP in d.w. also afforded protection against UVB-induced phosphorylation of ERK1/2 proteins in UVB irradiated skin.

A similar pattern of UVB-induced phosphorylation of JNK was observed when compared with the phosphorylation of ERK1/2 after multiple exposures of UVB in mouse skin (Figure 4B). Western blot analysis and subsequently densitometry of bands indicated that topical treatment of EGCG resulted in marked inhibition of UVB-induced phosphorylation of p46 (93%) and p54 (100%) of JNK as shown in Figure 4B. Treatment of GTP was found less protective than EGCG treatment, and afforded prevention of UVB-induced phosphorylation of p46 and p54 by 54 and 83%, respectively (Figure 4B). Administration of GTP in d.w. also resulted in inhibition of UVB-induced phosphorylation of p46 and p54 proteins by 39 and 17%, respectively, after multiple UVB exposures.

As was evident from western blot analysis and subsequently measurement of density of bands, multiple UVB exposures of mouse skin induced ~3-fold phosphorylation of p38 (Figure 4C). In non-UVB exposed mice, phosphorylated p38 was also detectable. As is evident from Figure 4C, topical treatment of EGCG or GTP prevented UVB-induced phosphorylation of p38 by 42% whereas GTP given in d.w. prevented only 9% compared with UVB alone irradiated group of mice. There was no difference in the total amount of p38 protein in UVB exposed and non-UVB exposed treatment groups (data not shown).

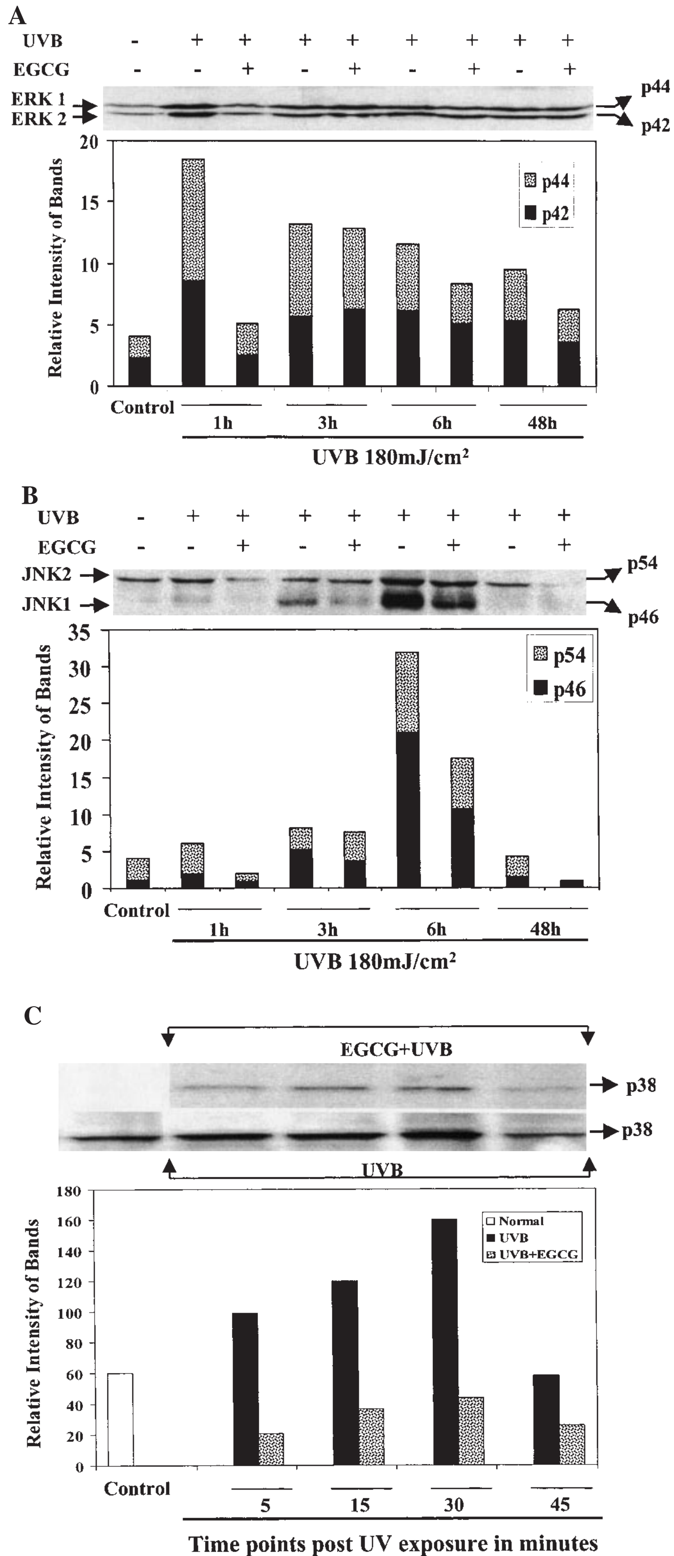

Fig. 3. (**A**) Topical treatment of EGCG prevents UVB-induced phosphorylation of ERK1/2 time-dependently *in vivo* in mouse skin. Mice were irradiated with a single dose of UVB (180 mJ/cm^2) with or without prior treatment with EGCG ($\approx$1 mg/cm^2 skin area) in hydrophilic cream. Mice were killed at different time points as shown in the figure after UVB exposure, skin biopsies were collected and epidermal lysates were prepared for western blot analysis to analyze phospho-specific proteins of MAPK as described in the Materials and methods section. Skin biopsies were pooled from two mice at each time point and in different treatment groups. Experiments were repeated three times with similar results. Maximum prevention of UVB-induced ERK1/2 by EGCG treatment was observed at 1 h after UVB irradiation. Treatments are as shown in the figure. Densitometric analysis was performed and relative intensity of bands is shown below the western blot. (**B**) Treatment of EGCG prevents UVB-induced phosphorylation of JNK time-dependently *in vivo* in mouse skin. Treatment protocol was similar as described in (A). Treatments are as labeled in the figure. Maximum induction of JNK was observed at 6 h after UVB irradiation. Densitometric analysis was performed and relative intensity of bands is shown below the western blot. Western blot and relative intensity of bands indicated that EGCG treatment resulted in marked inhibition of UVB-induced phosphorylation of JNK at 1, 6 and 48 h after UVB irradiation. (**C**) Treatment of EGCG prevents UVB-induced phosphorylation of p38 time-dependently *in vivo* in mouse skin. Treatment protocol was similar as described in (A). Treatments are as labeled in the figure. p38 is phosphorylated at earlier time points after UVB irradiation in comparison with ERK1/2 and JNK. The lower row of the western blot represents bands from UVB irradiation alone whereas the upper row of bands represents EGCG + UVB treatment. Densitometric analysis was performed and relative intensity of bands is shown below the western blot. Maximum induction of p38 was observed at 30 min after UVB irradiation. Western blot and relative intensity of bands indicated that topical treatment of EGCG resulted in marked inhibition of UVB-induced phosphorylation of p38 at every time point studied.

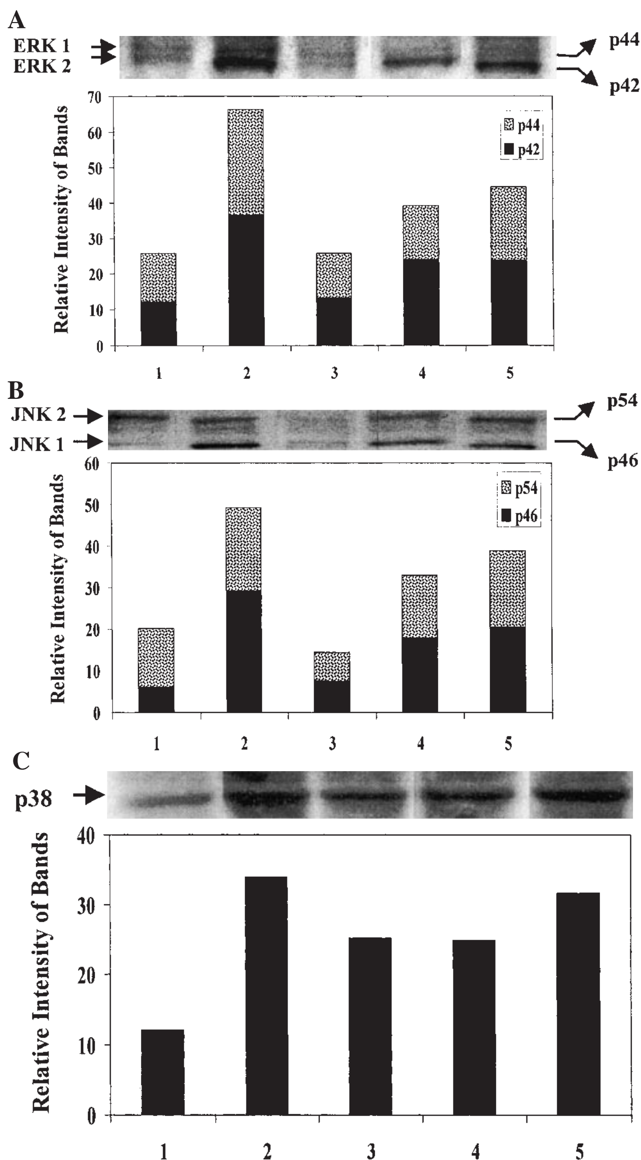

Fig. 4. (**A**) Treatment of green tea polyphenols prevents multiple UVB exposure-induced phosphorylation of ERK1/2 in *in vivo* mouse skin. Mice were UVB irradiated (180 mJ/cm^2) daily for total 10 consecutive days with or without treatment of EGCG and GTP. EGCG or GTP ($\approx$1 mg/cm^2 skin area) was topically applied in hydrophilic ointment 20–25 min before each UVB irradiation. GTP treatment was also given in d.w. (0.2%, w/v) 10 days prior to UVB irradiation and during UVB irradiation protocol. Mice were killed at 24 h after the last dose of UVB irradiation and skin biopsies were collected, and epidermal lysates were prepared for the western blot analysis to determine phospho-specific proteins of MAPK pathways as described in the Materials and methods section. Skin biopsies were pooled from two mice in different treatment groups to prepare lysate samples. Densitometric analysis was performed and relative intensity of bands is shown below the western blot. A representative blot is shown from three separate experiments with identical results. These results revealed that chemopreventive effect of EGCG (topical) > GTP (topical) > GTP (d.w.). Treatment groups were as follows: 1 = control (non-UV); 2 = UVB irradiation alone; 3 = EGCG + UVB; 4 = GTP + UVB; 5 = GTP in d.w. + UVB. (**B**) Treatment of green tea polyphenols prevents multiple UVB exposure-induced phosphorylation of JNK in *in vivo* mouse skin. Treatment protocol and groups were similar as described in (A). Western blots and relative intensity of bands indicated that topical treatment of EGCG is superior to green tea polyphenols treatment in terms of inhibition of UV-induced phosphorylation of JNK. (**C**) Treatment of green tea polyphenols prevents multiple UVB exposure-induced phosphorylation of p38 in *in vivo* mouse skin. Treatment protocol and groups were similar as described in (A). Western blots and relative intensity of bands indicated that topical treatments of EGCG and GTP prevented UVB-induced phosphorylation of p38.

Discussion

Non-melanoma skin cancer is the most frequently diagnosed cutaneous malignancy in the US and accounted for diagnosis of ~1.3 million new cases and 1200–1500 deaths each year (4). The role of dietary modification in reduction of skin cancer incidence has recently drawn widespread attention. It has been suggested that dietary intake of botanicals possessing substantial antioxidant properties could be a useful strategy to reduce the risk of non-melanoma skin cancer incidence. Diets rich in naturally occurring polyphenols like fruits and vegetables have been associated with the reduced incidence of certain human cancers (50–52). Among the polyphenolic antioxidants, the polyphenols from green tea have been shown to have strong anticarcinogenic effects (reviewed in refs 26–28), but their mechanism of protection is not yet well understood in the *in vivo* system particularly on UV-induced oxidative stress and oxidative stress-mediated cellular signaling pathways. In elucidating the chemopreventive mechanism of EGCG or GTP in *in vivo* animal model, we employed a hydrophilic ointment USP as a cream based formulation, which could be more appropriate for human use. Topical treatment of EGCG or GTP in hydrophilic ointment to SKH-1 hairless mice resulted in a significant prevention of single as well as multiple UV exposure-induced depletion of cutaneous endogenous antioxidant defence enzymes like GPx, catalase and GSH, and thus provides a possible mechanism of photoprotection (Figure 1). Antioxidant enzymes system function cooperatively. Any change in one of them may affect the equilibrium state of ROS or oxidative stress. Thus, if ROS remained without being scavenged in the biological system they may cause cellular damage and other biochemical alterations such as inflammation, lipid and protein oxidation, DNA damage and certain enzyme activation or inactivation (7,10–12,53,54). Therefore, prevention of UVB-induced depletion of antioxidant defence enzymes by EGCG or GTP treatment would provide sufficient protection against UVB-induced photodamage.

UVB-induced LPO and formation of protein carbonyls were used as markers of oxidative stress in the skin, and these were significantly inhibited by the treatment of polyphenols from green tea (Figure 2A). LPO in biological membrane is a free radical mediated event and is regulated by the availability of substrates in the form of polyunsaturated fatty acids, pro-oxidants which promote peroxidation and antioxidant defences such as α-tocopherol, GSH, β-carotene and superoxide dismutase (53–55). Elevated levels of LPO have been linked to injurious effects such as loss of fluidity, inactivation of membrane enzymes, increased permeability to ions and eventually rupture of the cell membrane leading to release of cell organelles (53–55). Peroxidation products can also result in damage to crucial biomolecules, including DNA (7,53,54). Thus, inhibition of UV-induced LPO levels by EGCG or GTP in the *in vivo* system should reduce the risk factors related to the UV-induced ROS-mediated tumor-promoting effects of UV radiation in cutaneous inflammatory responses and malignancies. The UV-induced oxidative stress-mediated protein oxidation may also be a detrimental factor for skin disorders. In this study, UV exposure of the skin increased protein oxidation by ~10- and 20-fold after single and multiple UV exposures, respectively, compared with non-UV exposed skin (Figure 2B). The inhibition of UVB-induced protein oxidation by GTP would result in the reduction of skin photodamage. To our knowledge this is for the first time that plant-derived

polyphenols, more specifically GTP, have been demonstrated to inhibit UVB-induced protein carbonyls formation in *in vivo* mouse skin. The exact mechanism of inhibition of protein oxidation by GTP is not clear but it can be suggested that EGCG or GTP may be preventing protein oxidation by scavenging free radicals, activating or enhancing the antioxidant defences of the skin, or activating the repair or proteolytic enzymes that repair or degrade damaged proteins (45).

MAPKs are important upstream regulators of transcription factor activities and their signaling is critical to the transduction of a wide variety of extracellular stimuli into intracellular events and thus thought to interplay in controlling the cellular events such as proliferation, differentiation and apoptosis in response to external signals or stimuli (48). Because EGCG has been shown to be the most potent antioxidant and major chemopreventive agent (26–28,31,35,36), first we determined the chemopreventive effect of topical treatment of EGCG on single UVB exposure-induced phosphorylation of MAPK proteins in *in vivo* animal model. Our data demonstrate that UVB-induced phosphorylation of proteins of MAPK family such as ERK1/2, JNK and p38 in *in vivo* mouse skin were prevented by the topical application of EGCG at different time points studied (Figure 3). However, their kinetics of activation after UVB irradiation are differed, for example ERK and p38 activation occurred at an early time points (within an hour) and JNK was found to activate at 6 h after UVB exposure (Figure 3). Moreover, differential activation of MAPK after UVB irradiation was observed in *in vivo* system (56,57). This discrepancy may depend on the stimulators used, the UV source and species and cell type under study. Treatment of EGCG *in vivo* in mouse skin inhibits UVB-induced phosphorylation of JNK. JNK regulates activator protein-1 (AP-1) transcription in response to environmental stress such as UV exposure (58). Increased AP-1 activity has been shown to be involved in the promotion and progression of various types of cancers and also other processes such as inflammation, invasion, metastasis and angiogenesis (16,17,59). Therefore, inhibition of AP-1 activation may be a relevant molecular target for potential chemopreventive agents (60). UVB-induced AP-1 activity was inhibited by EGCG in human keratinocytes (61). It has also been shown that EGCG treatment inhibited UVB-induced transcription of the *c-fos* gene and expression of the *c-fos* protein via mechanisms that appeared to involve inhibition of the p38, but not the ERK1/2 or JNK (61). These results suggest that treatment of EGCG affects MAPK proteins differently. It is well known that UVB induces H_2O_2 production in the target tissue, which in turn initiates phosphorylation of MAPK and activation of downstream signals and expression of genes having direct relevance in the process of carcinogenesis (18,62). Therefore, it can be suggested that treatment of EGCG prevented UVB-induced phosphorylation of MAPK by preventing UVB-induced oxidative stress such as depletion of antioxidant enzymes, and prevention of LPO and protein oxidation (Figures 1 and 2). Prevention of phosphorylation of MAPK proteins may thus prevent the down stream events such as activation of AP-1 and NF-κB, which would lead to the prevention of photocarcinogenic events in the skin. However, *in vitro* studies conducted in transfected human hepatoma HepG2 cells indicated that treatment of GTP activated MAPK proteins and followed different kinetics (63). This study suggested that the stimulation of MAPK could be the potential signaling pathways utilized by GTP to activate antioxidant-responsive element-dependent genes. Moreover, it may also be suggested that GTP may act differently in different model systems.

Treatments of EGCG and GTP were also found to prevent multiple UVB exposure-induced phosphorylation of MAPK proteins in *in vivo* mouse skin (Figure 4), thus indicating that polyphenols from green tea are equally effective against chronic exposure of UV irradiation. It is well documented that chronic exposure of UV radiation to skin leads to higher risk of photocarcinogenesis and other skin disorders. The data also demonstrate that chemopreventive efficacy of EGCG is superior to GTP treatment (Figure 4). GTP given in d.w. to mice also afforded significant protection against UVB-induced adverse biological effects. It is important to mention that topical treatment of EGCG and GTP to the skin may provide higher concentration or bioavailability of these ingredients to skin target cells whereas the bioavailability of GTP in the skin would be much less when it is given in d.w. Because of this reason, the photoprotective effects of GTP given in d.w. may be less in comparison with that of topical treatment. Further, this observation highlights the importance of GTP given in d.w. that oral consumption of GTP is also significantly photoprotective. This study also suggested that the use of hydrophilic cream for topical treatment of EGCG or GTP should be better and safer than the use of organic solvents like acetone with reference to human skin care.

Conclusively, this *in vivo* study further provides evidence that GTP and specifically EGCG has the potential to attenuate UVB-induced oxidative stress and oxidative stress-mediated cellular signaling of MAPK proteins, which are associated with the high risk of skin carcinogenesis. Based on the current observations, it appears that photoprotective effects of EGCG or GTP may be mediated by: (i) stimulation of endogenous antioxidant defence mechanisms, (ii) prevention of photodamage of macromolecules like lipids and proteins, and thus leads to, (iii) inhibition of phosphorylation of MAPK proteins. Because the early activation of cellular signaling pathways in response to UV irradiation is involved in the inflammatory reactions, photoaging, photodermatoses and photocarcinogenesis, the use of GTP as an antioxidant may have beneficial effects in protecting these cutaneous disorders in human population. Thus, it is tempting to suggest that green tea polyphenols may be supplemented as a pharmacologic agent in skin care products such as moisturizing creams, skin care lotions, facial and depilatory creams and sunscreens, etc., for the prevention and treatment of a variety of solar UV light-induced human skin disorders. However, extensive clinical trials or studies in *in vivo* human system are required to test the long-term treatment effects of polyphenols from green tea, their half-life period and optimum dose for beneficial effects.

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