Green Tea Polyphenols Prevent Ultraviolet Light-Induced Oxidative Damage and Matrix Metalloproteinases Expression in Mouse Skin

Praveen K. Vayali, * Anshu Mittal, * Yukihiro Hara, † Craig A. Elmets, †† and Santosh K. Katiyar* †††

*Department of Dermatology, University of Alabama at Birmingham, Birmingham, Alabama, USA; †Department of Environmental Health Sciences, University of Alabama at Birmingham, Birmingham, Alabama, USA; †Department of Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama, USA; †Center for Aging, University of Alabama at Birmingham, Birmingham, Alabama, USA; ‡Tokyo Food Techno Co. Ltd., Shizuoka, Japan

Chronic exposure of solar ultraviolet (UV) light to human skin results in photoaging. UV-induced oxidative damage and induction of matrix metalloproteinases (MMP) have been implicated in this process. Because polyphenols from green tea (GTP) prevent other cutaneous adverse effects of UV radiation we hypothesized that UV irradiation-induced oxidative damage and induction of MMP might be prevented in vivo in mouse skin by oral administration of GTP. GTP was administered in drinking water (0.2%, wt/vol) to SKH-1 hairless mice, which were then exposed to multiple doses of UVB (90 mJ per cm², for 2 mo on alternate days) following in vivo photoaging animal protocol. Treatment of GTP resulted in inhibition of UVB-induced protein oxidation in vivo in mouse skin, a hallmark of photoaging, when analyzed biochemically, by immunoblotting, and immunohistochemistry. GTP treatment also inhibited UVB-induced protein oxidation in vitro in human skin fibroblast HS68 cells, which supports in vitro observations. Moreover, oral administration of GTP also resulted in inhibition of UVB-induced expression of matrix degrading MMP, such as MMP-2 (67%), MMP-3 (63%), MMP-7 (62%), and MMP-9 (60%) in hairless mouse skin. These data suggest that GTP as a dietary supplement could be useful to attenuate solar UVB light-induced premature skin aging.

Key words: green tea polyphenols/matrix metalloproteinase/photoaging/protein oxidation/ultraviolet light


Chronic exposure of human skin to solar ultraviolet (UV) radiation is a major environmental factor that has serious adverse effects on the structure and function of the skin. Depending on the amount and form of the UV radiation, as well as on the skin type of the individual exposed, UV irradiation may cause sunburn, immunosuppression, oxidative stress, non-melanoma and melanoma skin cancers as well as premature aging of the skin so-called photoaging (Kligman, 1986; Brenneisen et al., 2002). In humans, photoaging is characterized by dry and flaky rough skin, both fine and coarse wrinkles, poor elastic recoil, solar lentigines, sallow color and impaired wound healing (Kligman, 1986). There is increasing evidence for the generation of reactive oxygen species (ROS) in skin upon UV exposure (Katiyar and Mukhtar, 2001a; Katiyar and Elmets, 2001b). Increased ROS generation can overwhelm antioxidant defense mechanisms, resulting in oxidative stress and oxidative photodamage of proteins and other macromolecules in the skin. Although cellular and mitochondrial defense mechanisms including the antioxidant enzymes have evolved to quench ROS, these antioxidant defense systems are not fully efficient, and hence throughout life cells accumulate molecular oxidative damage to lipid, protein and DNA, which may lead to an age-associated increase and eventually may lead to photoaging of the skin (Fuchs et al., 1989; Pence and Naylor, 1990; Young, 1990; Shindo et al., 1994). Photoaging is thought to occur because of continuous damage to the collagenous extracellular matrix that comprises the dermal connective tissue. Recently it was demonstrated in human skin that acute and chronic photodamage is mediated by depleted antioxidant enzyme expression and increased oxidative protein modifications (Sander et al., 2002) and accumulation of lipid peroxidation and glycation products (Jeanmaire et al., 2001; Tanaka et al., 2001, Fisher et al., 2002). Previous studies in hairless mice using iron-chelators and antioxidants indicate that UV-induced ROS are involved in photoaging (Bissett et al., 1990; Bissett and McBride, 1996). It has also been shown that exposure of human or mouse skin to UV irradiation upregulates the synthesis of the matrix-degrading enzymes matrix metalloproteinases (MMP), such as MMP-1, -2, -3, -7, -8, -9, and -12 (Fisher et al., 1998, 2001; Fisher and Voorhees, 1998; Saarialho-Kere et al., 1999; Inomata et al., 2003), which have been implicated in photoaging.

In recent years, polyphenols from green tea (GTP) have attracted considerable attention because of their skin

Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; EGCG, (−)-epigallocatechin-3-gallate; GTP, green tea polyphenols; MMP, matrix metalloproteinase; ROS, reactive oxygen species; UV, ultraviolet
photoprotective effects (Katiyar et al, 2000; Katiyar and Elmets, 2001b). Green tea (Camellia sinensis) contains polyphenols, which have gained great interest due to their potent antioxidant activities (Katiyar and Elmets, 2001b). GTP have been shown to have remarkable preventive effects against phototoxicity in murine models as well as in humans (Katiyar and Elmets, 2001b, Katiyar et al, 2001c). Oral feeding of GTP to SKH-1 hairless mice as a sole source of drinking water followed by UV irradiation resulted in significant protection against UV-induced cutaneous edema and inhibition of cyclooxygenase activity and their metabolites (Agarwal et al, 1993). Topical application of GTP to mouse skin before UV exposure decreased the UV-induced hyperplastic response, edema, myeloperoxidase activity and suppression of contact hypersensitivity (Katiyar et al, 1995). Studies have recently shown that treatment of (−)-epigallocatechin-3-gallate (EGCG), a polyphenolic constituent of green tea, even without UV exposure directly inhibits the expression of MMP such as MMP-9, MMP-2, MT1-MMP and neutrophil elastase at pharmacologically achievable concentrations (Benelli et al, 2002; Dell’Aica et al, 2002). Therefore, we hypothesized that oral administration of GTP in drinking water will prevent the markers of cutaneous photoaging such as inhibition of UVB-induced oxidative damage of proteins and expression of MMP. To test this hypothesis we conducted experiments in a hairless mouse model of photoaging (Kligman, 1996) where mice were exposed to multiple exposures of UVB to determine whether GTP has protective effects on photoaging. Photoprotective effects of GTP were determined on UVB-induced oxidative damage of proteins, and expression of MMP, such as MMP-2 (gelatinase A), MMP-7, MMP-9 (gelatinase B) that degrades collagen fragments of type I and III generated by collagenase, and MMP-3 (stromelysin) that degrades type IV collagen of the basement membrane (Rittie and Fisher, 2002).

**Results**

**Oral administration of GTP and its consumption** During the experimental period each mouse (approximately 25 g in weight) consumed 5–6 mL of drinking water per day with or without GTP. Thus, an average each mouse consumed 10–12 mg of GTP per day. The consumption of drinking water with or without GTP was monitored throughout the experimental protocol, and no significant variation in its consumption was observed. To avoid light-induced changes in the polyphenols, the bottles containing drinking water were covered with aluminum foils. Under these conditions at room temperature (24 ± 2°C), the green tea polyphenols are not significantly oxidized or degraded. Very limited studies on bioavailability of green tea polyphenols in skin in vivo have been conducted. Acute administration of EGCG by gastric tube was performed in mice, which showed the presence of EGCG metabolites in all target organs including the skin of mice (Suganuma et al, 1998). This observation, however, was different from this study where GTP was given to mice in drinking water.

**Treatment of HS68 fibroblast cells in vitro with GTP inhibits UVB and H2O2-induced protein oxidation** Because most of the abnormalities present in photoaged skin are caused by oxidative damage to proteins present in the dermis and produced by fibroblasts, initial studies were conducted to determine whether GTP protected against UV-induced protein oxidation in skin fibroblasts. For this purpose, we employed an in vitro system in which the human skin fibroblast cell line HS68 was exposed to UVB radiation (30 mJ per cm²). HS68 cells treated with UVB significantly induced protein oxidation (Fig 1). The addition of GTP to HS68 cells concentration dependently inhibited UVB-induced protein oxidation (Panel A). As a positive control for these experiments the HS68 cell line was treated with H2O2 instead of UVB. As shown in Fig 1 (Panel B), treatment with GTP markedly inhibited H2O2 (25 μM)-induced protein oxidation as well. Since GTP plays a role as an antioxidant, we used an endogenous antioxidant, catalase, to determine its effect on H2O2-induced oxidation of proteins. Treatment with catalase produced results to those of GTP with respect to inhibition of H2O2-induced protein oxidation in HS68 cells (Panel C).

**Oral administration of GTP inhibits UVB-induced histopathological changes in the skin** Photoaging of the skin is caused by chronic UV exposure, which results in atrophy of the epidermis and damage to dermal proteins. There have been no studies that have examined the photoprotective effect of GTP on photoaging following chronic UV radiation exposure. To examine the photoprotective effect of GTP on the histopathologic changes in the skin that occur following chronic UVB exposure in vivo, SKH-1 hairless mice were treated with UVB (90 mJ per cm²) for 2 mo on alternate days. The histopathological changes in the skin were analyzed by H & E staining. In the normal non-UV exposed skin (Fig 2, top panel), the epidermis was observed to be thin with a thickness of two to three cell layers. A hyperplastic response was evident in skin that had been treated with the chronic UVB regimen and was six to eight cell layers thick (Fig 2, middle panel). Epidermal thickness was measured as 15 ± 4, 46 ± 7 and 25 ± 5 μm in normal (non-UV exposed), UVB and GTP + UVB exposed skin, respectively. Thus treatment of GTP to UVB exposed mice reduced the epidermal hyperplastic response by 67% (p < 0.001). In chronic UVB irradiated dermis (Fig 2, middle panel), the sebaceous glands were enlarged, there was proliferation of dermal cysts and there were a large number of vacuoles in comparison with non-UV exposed normal skin. Oral feeding of GTP to mice prevented these morphological changes (Fig 2, bottom panel) indicating that GTP had a photoprotective effect on both the epidermis and dermis.

**Oral administration of GTP inhibits UVB-induced protein oxidation** It has been shown that oxidatively modified proteins accumulate specifically within the upper dermis of photoaged skin (Sander et al, 2002). We were, therefore, interested in determining whether carbonyl levels, a measure of protein oxidation, increased in the skin of mice exposed to chronic UVB irradiation. We observed that chronic UVB irradiation induced high levels of protein carbonyls by approximately 21-fold compared with that of non-UV exposed control animals (Fig 3, Panel A). Oral
feeding of GTP in drinking water significantly protected skin from protein carbonylation (50%, p < 0.001) after chronic UVB exposure. Formation of protein carbonylation by UVB exposure was corroborated by western blot analysis (Fig 3, Panel B) of tissue extracts using a specific antibody against dinitrophenylhydrazone. Skin lysates prepared from UVB irradiated skin showed higher levels of protein oxidation after chronic UVB compared with non-UVB exposed skin (Fig 3, Panel B). GTP treatment alone did not induce protein oxidation compared with that of non-GTP-treated control mice (Fig 3, Panels A and B). Densitometric analysis of bands indicated that oral feeding of GTP to mice inhibited UVB-induced oxidation of proteins in the skin by 37% (p < 0.01) following chronic exposure to UV radiation. The intensity of bands in different treatment regimens is shown in terms of protein carbonyl content (% of control) (Fig 3, Panel C).

Immunostaining was performed to localize oxidized proteins in the skin. Chronic UVB exposure induced higher levels of protein oxidation within the dermis relative to the epidermis (Fig 4, middle panel). Both were greater than that
Oral administration of GTP inhibits UVB-induced protein oxidation in mouse skin. Oral administration of GTP in drinking water inhibited UVB-induced protein oxidation in the dermis as indicated by decreased staining intensity (bottom panel). These observations provide additional evidence that GTP have a photoprotective effect against protein oxidation.

Oral administration of GTP inhibits UVB-induced expression of MMP. Photoaged skin has been shown to contain elevated levels of MMP and they have been considered to be markers of photoaging. We were interested in evaluating the photoprotective effect of GTP on the expression of different MMP that developed in mouse skin following chronic UVB exposure. Densitometric analysis of bands from western blots indicated that chronic UVB exposure induced high levels of MMP-2 (3-fold), MMP-3 (10-fold), MMP-7 (2-fold), and MMP-9 (2-fold) compared with that of non-UVB exposed mouse skin, as shown in Fig 5. Administration of GTP in drinking water was found to prevent UVB-induced expression of MMP-2 (Panel D), MMP-3 (Panel C), MMP-7 (Panel B), and MMP-9 (Panel A) by 67%, 62%, 63%, and 60%, respectively, in the
Discussion

Photo-oxidative damage caused by solar UV light is the leading cause of extrinsic aging of the skin. Photoaging causes numerous histologic, physiologic, and clinical changes (Kligman, 1986). We showed that acute exposure of solar UV irradiation on the skin induces oxidative stress (Katiyar and Mukhtar, 2001a; Katiyar and Elmets, 2001b; Katiyar et al., 2001c), and oxidative stress is considered to be a major contributor to the process of photoaging of the skin (Harman, 2001; Rittie and Fisher, 2002). Although cytoplasmic and mitochondrial defense mechanisms including the antioxidant enzymes glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase have evolved to quench ROS, these antioxidant defense enzymes are not fully efficient, and hence throughout life cells accumulate molecular evidence of oxidative damage. Several studies have shown that there is an age-associated increase in both ROS generation (Sohal and Brunk, 1992) and the level of oxidatively damaged proteins (Stadtman and Levine, 2000). Therefore new chemopreventive agents and strategies need to be developed that can prevent solar UV light-induced oxidative damage, and oxidative damage mediated photoaging process. One of the approaches that has generated enormous interest is the use of antioxidants (Fisher et al., 1999; Sander et al., 2002). Diets rich in naturally occurring polyphenols, primarily from fruits and vegetables, have been associated with a reduced incidence of ROS-mediated diseases. On photodamaged skin, it has been reported that vitamin A, E, C, and procyanidins exhibit photoprotective effects on skin due to their antioxidant activities (Eberlein-Konig et al., 1998; Stahl et al., 2000; Mittal et al., 2003). In this context, there is great interest in using dietary botanical supplements for the prevention of photoaging.

This *in vivo* study demonstrates that oral administration of GTP provided protection against the UVB-induced damaging effects on skin. The skin possesses a wide range of interlinked antioxidant defense mechanisms to protect itself from damage by UVB-induced oxidative stress. The capacity of these systems, however, is limited and they can be overwhelmed by excessive exposure to UV resulting in damage to target cells. Oral administration of GTP as a sole source of drinking water significantly protected UVB-induced oxidation of protein macromolecules in the skin compared with that of non-GTP-treated mice. Studies have found increased levels of oxidized proteins in keratinocytes from aged donors (Petropoulos et al., 2000). Other studies have shown that protein carbonyl formation is most pronounced in the dermis after UV irradiation, where the major pathologic changes of photoaging occur (Sander et al., 2002). In this study, we found that GTP inhibited the elevated levels of protein carbonyls that occurred in the skin after chronic UVB exposure. Most cellular signaling pathways involved in photoaging are mediated through oxidative stress (Katiyar and Elmets, 2001b; Rittie and Fisher, 2002). It has been shown that depletion of antioxidant enzymes level in photodamaged skin also associated with higher levels of protein oxidation (Sander et al., 2002). We also found that oral administration of GTP to mice resulted in prevention of UVB-induced depletion of antioxidant enzymes such as...
glutathione peroxidase, catalase and the level of glutathione (Vayalil et al., 2003; and unpublished observations). GTP may therefore be preventing protein oxidation by initiating a cascade of events in which decreased oxidative stress in the skin, and/or activated repair or proteolytic enzymes that repair or degrade damaged proteins could be involved (Stadtman and Levine, 2000). Treatment of GTP in vitro to human skin fibroblast HS68 cells resulted in inhibition of UVB and H₂O₂ (used as an oxidative agent) induced oxidation of proteins. The treatment of catalase, an endogenous antioxidant, to HS68 cells also resulted in inhibition of H₂O₂ induced oxidation of proteins, thus indicating the role of GTP as an antioxidant, and supporting our findings in in vitro system. Currently, the dietary chemopreventive agents that may prevent protein oxidation in vivo are limited. To our knowledge, this is the first detailed report to show that polyphenols from green tea inhibit UV-induced protein carbonylation or protein oxidation in vivo.

Photoaging is characterized by degradation of collagen and accumulation of abnormal elastin in the superficial dermis; several matrix MMP have been implicated in this process (Saarialho-Kere et al., 1999; Fisher et al., 2002; Inomata et al., 2003). Latent MMP-1, -7, and -9 derived from keratinocytes or dermal fibroblasts could be maximally activated by MMP-3, and ultimately initiate degradation of collagen types I and III. Simultaneous expression of MMP-2, -3, -7, and -9 could lead to degradation of non-collagenous extracellular matrix, including the basement membrane and proteoglycans (Kahari and Saarialho-Kere, 1997; Fisher and Voorhees, 1998). Our study in this photoaging model demonstrates that chronic exposure of mouse skin to UVB-induced expression of MMP-2, -3, -7, and -9, all of which have been shown to be involved in the degradation of types-I and -III collagen fragments generated by collagenases, and type IV collagen of the basement membrane (Rittle and Fisher, 2002). Oral administration of GTP markedly inhibited UV-induced expression of these MMP in vivo mouse skin suggesting that GTP has a potential anti-photoaging effect. Treatment of EGCG, a major polyphenolic constituent of green tea, has been shown to reduce UVA-induced skin damage (roughness and sagginess) and protected from the decrease of dermal collagen in hairless mouse skin, and also blocked the UV-induced increase of collagen secretion and collagenase mRNA level in fibroblast culture (Kim et al., 2001). In another study, feeding of green tea extract to Sprague–Dawley rats remarkably inhibited the age-associated increase in the fluorescence in the aortic collagen, whereas that occurring in the skin collagen was not significantly inhibited by this treatment (Song et al., 2002). It should be noted that the difference in the observations of Song et al (2002) and those presented here may relate to the fact that they were examining changes in skin collagen that had not been subjected to UV exposure, whereas the changes in this series of experiments, the skin had received chronic UV treatments over a period of 2 mo. Moreover, since this preparation of GTP contains only traces of caffeine (<0.3%), we do not expect that caffeine has any significant role in prevention of UV-induced adverse effects in this study.

Further, in vivo tissue distribution of GTP was not adequately studied after oral administration, particularly in skin tissues, therefore the link between GTP and biological effects are not clearly understood. Kim et al (2000) demonstrated the tissue distribution of GTP in lungs and liver of mice after oral uptake of GTP. This study concluded that consumption of tea by rodents could induce adaptive responses affecting blood and tissue levels of tea catechins with time. It may also possible that GTP are not directly affecting the skin cells in vivo, but could have indirect effects through cells in other tissues. Again it is suggested that GTP metabolites reached the skin tissues or cells and results in protection against adverse effects of UV radiation. More appropriately, however, the mechanism by which GTP is affecting UV-induced responses in the skin in vivo is unclear at this time.

The data obtained in this study provide evidence that the anti-photoaging effect of GTP in vivo in skin is mediated, at least, by (i) reduction in oxidative damage of the protein macromolecules, and (ii) inhibition of expression of MMP that degrade extracellular matrix proteins. Green tea polyphenols may thus be useful candidate for prevention against solar UV light-induced premature aging of the skin in humans. Detailed mechanistic and clinical trials, however, are warranted to test the potential benefits of green tea polyphenols on photaging of the skin in humans.

### Materials and Methods

**Animals**  Pathogen-free female SKH-1 hairless mice (6–7-wk old) were purchased from Charles River Laboratory (Wilmington, Massachusetts) and were housed in our pathogen-free barrier animal facility at the University of Alabama at Birmingham. Mice were kept in groups of four per cage and fed with Teklad chow diet and water ad libitum. The animals were acclimatized for at least 1 wk before the start of experiments, and were maintained at a 12 h dark/12 h light cycle, 24°C ± 2°C temperature and 50% ± 10% relative humidity. The animal protocol for this study was approved by Institutional Animal Care and Use Committee of the University of Alabama at Birmingham, in accordance with the current US Department of Agriculture, Department of Health and Human Service regulations and standards.

**Human fibroblast HS68 cells culture and treatment** Human foreskin fibroblast HS68 cells were obtained from American Type Culture Collection (Rockville, Maryland) to determine the in vitro effect of GTP on UVB- or H₂O₂ (an oxidant)-induced protein oxidation. Cells were grown in Dulbecco's modified eagle's medium (DMEM) with 4 mM L-glutamine and 4.5 g per L glucose, supplemented with fetal bovine serum (10%, vol/vol) and penicillin (100 U per mL) and streptomycin (100 mg per mL) in an incubator at 37°C in a humidified atmosphere containing 5% CO₂.

**Antibodies and reagents** Primary antibodies to MMP-2, -3, -7, and -9 were purchased from SantaCruz Biotechnologies (Santa Cruz, California). Purified GTP (containing >86% epicatechin derivatives) was obtained from Tokyo Food Techno Co. Ltd., Shizuoka, Japan. Diaminobenzidine reagent set was purchased from Kirkggaard and Perry (Gaithersburg, Maryland). OxyBlot protein oxidation western blot detection kit was purchased from Intergen Company (Purchase, New York). All other chemicals and reagents used in this study were of highest grade and purity.

**UV irradiation** UV irradiation was performed as described previously (Katiyar and Mukhtar, 2001a). Briefly, the dorsal skin was exposed to UV irradiation from a bank of four FS-20 fluorescent lamps from which wavelengths (<290 nm) not normally present in natural solar radiation were filtered out using Kodacel
After filtration with a Kodacel film, the majority of the resulting wavelengths of UV radiation were in the UVB (290–320 nm) and UVA (320–400 nm) range with a peak emission at 314 nm as monitored. The UVB emission was monitored with an IL-1700 phototherometry radiometer equipped with an IL SED 240 detector fitted with a W side angle quartz diffuser and an SC5 280 filter (all from International Light, Newburyport, Massachusetts). During UVB irradiation mice were housed in specially designed cages where they were held in dividers separated by Plexiglas. Dorsal skin of mice was exposed to chronic UVB regimen consisting of multiple UVB exposures (90 mJ per cm²) for 2 mo on alternate days.

**GTP treatment** Purified GTP is a mixture of five major catechin derivatives. It was obtained from Tokyo Food Techno Co., Ltd., Shizuoka, Japan. The major five constituents found in GTP are: (→)-epicatechin (10.4%), (→)-epigallocatechin (8.3%), (EGCG) (55.8%), (→)-gallocatechin gallate (4.2%) and (→)-epicatechin gallate (6.9%). This preparation of GTP contains traces of caffeine (<0.3%). GTP (0.2%, wt/vol) was given orally as a sole source of drinking water 10 d before the start of UVB irradiation and was continued until 24 h after the last UVB exposure. GTP containing water was regularly changed twice a week, and GTP containing water bottles were covered with aluminum foil to avoid light-induced oxidation of polyphenols, if any. The control groups, with or without UVB irradiation, were given drinking water alone without GTP.

**Histology of the skin** To evaluate the effect of GTP on UV-induced changes in skin morphology, mice were sacrificed 24 h after the last UV exposure. Skin biopsies were obtained from the dorsal skin, perpendicular to the long axis of the trunk, fixed in 10% buffered formalin and processed for H & E staining for microscopic evaluation.

**Preparation of skin lysates** The skin biopsies were collected and pooled from each mouse in each treatment group. The subcutaneous tissue was removed using a shaving scalpel, and the skin was washed several times with 0.05 M Tris–HCl buffer, pH 7.4, containing 0.15 M NaCl. The skin samples were homogenized using a Polytron homogenizer in ice-cold lysis buffer containing 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 NaN₃; and aprotinin and leupeptin 1 µg per mL each). Homogenates were centrifuged at 14,000 g for 20 min at 4 °C. The clear supernatants were used for analysis of protein carboxyls, and for the western blot analysis of protein carboxyls and MMP.

**Assay for protein carboxyls or protein oxidation** The quantitative analysis of protein carboxyls was performed by using 2,4-dinitrophenylhydrazine (DNPH) as described previously (Gao and Cutter, 1995; Levine et al, 2000). Briefly, the nucleic acids present in the skin lysates that contains carboxyl groups reactive with DNPH were initially precipitated out using streptomyacin sulfate (1% final concentration) followed by dialysis against water for 2.5 h and water was changed once after 1.5 h. The precipitate was removed by centrifugation at 14,000 g at 4 °C. To 1 mL of the above supernatant (containing ~1–1.5 mg per mL protein) 4 mL of 12.5 mM DNPH in 2.5 M HCl or 2.5 M HCl alone (blank) was added and incubated at room temperature for 1 h. The protein was precipitated with 10% trichloroacetic acid. The pellet was washed 3–5 times by breaking the pellet with a glass rod to remove the free DNPH with 4 mL of ethanol:ethyl acetate (1:1, vol/vol). The pellets were dissolved in 6 M guanidine–HCl at 37 °C for 20 min with frequent vortexing. Insoluble materials were removed by centrifugation and absorbance was measured at 370 nm. The protein carboxyl content was calculated from the molar absorption coefficient (ε) of 22,000 M⁻¹ cm⁻¹. The protein content was determined by using BioRad protein detection kit following the manufacturer’s protocol.

**Western blot analysis of protein oxidation** For western blot analysis of protein oxidation, the OxyBlot Protein Oxidation Detection kit (Intergen, Company, Purchase, New York) was employed following the manufacturer’s protocol. The protein lysate (5 µg) was denatured by adding sodium dodecyl sulfate to a final concentration of 6% and derivatized using DNPH solution for 15 min. Negative control samples were simultaneously treated with derivatization control solution. Reactions were stopped by neutralizing solution. The derivatized samples were loaded into the 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted onto a nitrocellulose membrane. The membrane was blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% non-fat dry milk for 1 h. The membrane was then incubated overnight at 4 °C with the corresponding diluted primary antibody in non-fat dry milk in TBS-T as described by the manufacturer. This was followed by incubating the membrane with HRP-conjugated secondary antibody for 1 h at room temperature in TBS-T containing 5% non-fat dry milk. The membrane was washed in TBS-T and treated with a chemiluminescence reagent ECL detection kit (Amersham, Piscataway, New York) according to the manufacturer’s protocol. The bands were visualized by exposing the membrane to XAR-5 film (Eastman Kodak Co., Rochester, New York). The intensity of protein carboxylation was determined by densitometry of the bands on the autoradiograph using OPTIMAS 6.2 computerized program. After developing, blots were stained with Coomassie Blue to confirm equal loading of proteins.

**Histochromelal detection of carboxylated proteins** As a marker of ROS-mediated protein oxidation and oxidative stress, protein carboxyls were detected by the DNPH method using OxyBlot Protein Oxidation Detection Kit (Intergen, Purchase, New York) as described previously (Sander et al, 2002) with some modifications. Five µm thick frozen skin sections were fixed in cold acetone and thereafter incubated with DNPH solution (diluted 1:100) or control derivatization solution (1:100) for 1 h. After three successive washings for 5 min each, the sections were blocked using 3% BSA in PBS for 30 min, and incubated with rabbit anti-dinitrophenylhydrazone antibody (Intergen, Purchase, New York) in PBS for 1 h at 37 °C. Endogenous peroxidase was blocked with 0.5% hydrogen peroxide in PBS for 30 min. After washing, the sections were incubated with biotinylated goat anti-rabbit secondary antibody for 1 h at room temperature. Sections were washed and incubated again with peroxidase-conjugated streptavidin for 30 min. After washing, the reaction product was visualized after incubating with diaminobenzidine and then washed with distilled water. Sections were counterstained with methyl green. For a negative control, sections were treated with DNPH solution instead of control derivatization solution.

**Western blot analysis of MMP** For immunoblotting of MMP, 50–80 µg of protein in Laemml’s buffer was loaded on 10% polyacrylamide gels and was run at a constant voltage of 125 V until the tracking dye reached 1 cm from the bottom of the gel. This was followed by electrophotography onto a nitrocellulose membrane for 2 h at constant voltage of 25 V. The rest of the procedure was identical to that described for Western blot analysis of protein carboxylation except for the specific primary and secondary antibodies to MMP-2, -3, -7, and -9. The intensity of protein bands was determined by densitometry of the bands on the autoradiograph using OPTIMAS 6.2 computerized program.

**Statistical analysis** The results of antioxidant enzymes and protein carboxylation are expressed as means ± SD in terms of percent of control. Statistical analysis of all data between the UV irradiated and GTP + UV irradiated groups was analyzed by Student’s t-test. A p value <0.05 was considered statistically significant. Immunohistochemical staining of sections for protein oxidation was analyzed using densitometric image analysis. At least three different areas from epidermis and dermis of each
section were selected and the intensity of staining for a total area was measured and quantified using the OPTIMAS 6.2 computerized program.

This work was supported by the funds from the National Institute of Environmental Health Sciences (ES11421), National Cancer Institute (CA94593), and Purdue/UAB Botanical Center for Age-Related Diseases (S.K.K.), and also by funds from the National Cancer Institute (CA79200) and the funds from the Veterans Administration (18-103-02, C.A.E.).

DOI: 10.1111/j.0022-202X.2004.22622.x

Manuscript received August 13, 2003; revised February 7, 2004; accepted for publication February 24, 2004

Address correspondence to: Santosh K. Katiyar, Ph.D., Department of Dermatology, University of Alabama at Birmingham, 1670 University Boulevard, Volker Hall 557, PO Box 202, Birmingham, AL 35294, USA. Email: skatiyar@uab.edu

References


Dell’Aica I, Dona M, Sartor L, Pezzato E, Garbisa S, Brenneisen P, Sies H, Scharffetter-Kochanek K: (CA79820) and the funds from the Veterans Administration (18-103-02, C.A.E.).

Email: skatiyar@uab.edu

Manuscript received August 13, 2003; revised February 7, 2004; accepted for publication February 24, 2004

Address correspondence to: Santosh K. Katiyar, Ph.D., Department of Dermatology, University of Alabama at Birmingham, 1670 University Boulevard, Volker Hall 557, PO Box 202, Birmingham, AL 35294, USA. Email: skatiyar@uab.edu

References


Dell’Aica I, Dona M, Sartor L, Pezzato E, Garbisa S, Brenneisen P, Sies H, Scharffetter-Kochanek K: (CA79820) and the funds from the Veterans Administration (18-103-02, C.A.E.).

Email: skatiyar@uab.edu

Manuscript received August 13, 2003; revised February 7, 2004; accepted for publication February 24, 2004

Address correspondence to: Santosh K. Katiyar, Ph.D., Department of Dermatology, University of Alabama at Birmingham, 1670 University Boulevard, Volker Hall 557, PO Box 202, Birmingham, AL 35294, USA. Email: skatiyar@uab.edu

References


Dell’Aica I, Dona M, Sartor L, Pezzato E, Garbisa S, Brenneisen P, Sies H, Scharffetter-Kochanek K: (CA79820) and the funds from the Veterans Administration (18-103-02, C.A.E.).

Email: skatiyar@uab.edu

Manuscript received August 13, 2003; revised February 7, 2004; accepted for publication February 24, 2004

Address correspondence to: Santosh K. Katiyar, Ph.D., Department of Dermatology, University of Alabama at Birmingham, 1670 University Boulevard, Volker Hall 557, PO Box 202, Birmingham, AL 35294, USA. Email: skatiyar@uab.edu

References


Dell’Aica I, Dona M, Sartor L, Pezzato E, Garbisa S, Brenneisen P, Sies H, Scharffetter-Kochanek K: (CA79820) and the funds from the Veterans Administration (18-103-02, C.A.E.).

Email: skatiyar@uab.edu

Manuscript received August 13, 2003; revised February 7, 2004; accepted for publication February 24, 2004

Address correspondence to: Santosh K. Katiyar, Ph.D., Department of Dermatology, University of Alabama at Birmingham, 1670 University Boulevard, Volker Hall 557, PO Box 202, Birmingham, AL 35294, USA. Email: skatiyar@uab.edu