Inhibition of UVB-mediated Oxidative Stress and Markers of Photoaging in Immortalized HaCaT Keratinocytes by Pomegranate Polyphenol Extract POMx

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ABSTRACT

In recent years there has been an increase in use of botanicals with antioxidant properties as skin photoprotective agents. Pomegranate (Punica granatum L.) fruit possesses strong antioxidant and anti-inflammatory properties. Recently, we have shown that pomegranate-derived products rich in anthocyanins and ellagitannins inhibit UVB-mediated activation of nuclear factor kappa B and modulate UVA-mediated cell proliferation pathways in normal human epidermal keratinocytes. In this study, we evaluated the effect of polyphenol-rich pomegranate fruit extract (POMx) on UVB-induced oxidative stress and photoaging in human immortalized HaCaT keratinocytes. Our data show that pretreatment of HaCaT cells with POMx (10–40 gm L–1) inhibited UVB (15–30 mJ cm–2)-mediated (1) decrease in cell viability, (2) decrease in intracellular glutathione content and (3) increase in lipid peroxidation. Employing immunoblot analysis we found that pretreatment of HaCaT cells with POMx inhibited UVB-induced (1) upregulation of MMP-1, -2, -7 and -9, (2) decrease in TIMP-1, (3) phosphorylation of MAPKs and (iv) phosphorylation of c-jun, whereas no effect was observed on UVB-induced c-fos protein levels. These results suggest that POMx protects HaCaT cells against UVB-induced oxidative stress and markers of photoaging and could be a useful supplement in skin care products.

INTRODUCTION

Ultraviolet (UV) radiation from the sun is associated with many skin disorders in humans and induces a number of harmful responses including erythema, edema, hyperpigmentation, hyperplastic responses, immunosuppression, photoaging and skin cancer (1–3). UVB and to a lesser extent UVA are causative factors for sunlight-induced skin disorders diagnosed in humans. UVB radiation is the most damaging component of the solar radiation reaching the earth and acts mainly on the epidermal basal cell layer of the skin. Exposure of the skin to UV light initiates a photo-oxidative reaction which impairs the antioxidant status and increases the cellular level of reactive oxygen species (ROS) accompanied by activation of many ROS-sensitive signaling pathways (3–5). This impairs the ability of the skin to protect itself from excessive generation of ROS resulting in increased oxidative stress, with consequent damage to the cutaneous tissues, a process commonly known as ‘photoaging.’

Skin aging is a complex phenomenon that occurs from interactions between intrinsic (genetic) and extrinsic (environment and lifestyle) factors. Intrinsic or chronological aging is an inevitable, genetically programmed process related to a natural decline of the normal physiological process whereas extrinsic aging is caused due to insult from the environment. Both intrinsic and extrinsic factors are superimposed in the sun-exposed areas of skin, resulting in deleterious effects (6,7). Chronic exposure to UV radiation results in marked histological changes in the extracellular matrix (ECM), reflecting a clinically photoaged skin with loss of rigidity and elasticity. The skin appears rough and leathery with deep coarse wrinkles and uneven pigmentation on the surface. It is known that altered synthesis of ECM proteins as well as their degradation by matrix metalloproteinases (MMPs) contribute to the dermal remodeling of the photoaged skin (6). MMPs are also known to play important roles in tissue destruction during pathological processes, such as arthritis, skin aging, tumor invasion and metastasis. MMPs are induced by various extracellular stimuli, e.g. UV or infrared radiation, growth factors, cytokines and tumor promoters (9), and are assumed to be involved in the degradation of the epidermal basement membrane and wrinkle formation (10).

Supplementing antioxidants has been reported to repair skin damage, loss of elasticity, wrinkling and premature aging. Antioxidants have the capability to quench ROS and studies show that antioxidants can inhibit many UV-induced signal transduction pathways (11). Thus it is suggested that a regular intake of dietary antioxidants or treatment of skin with antioxidant-containing natural ingredients may be a useful strategy for prevention of UV-mediated cutaneous damage. Polyphenolics, widely distributed in botanicals, form an integral part of human diet with significant amount of it present in vegetables, fruits and beverages and possess strong antioxidant and free radical scavenging activity (12,13). Pomegranate (Punica granatum L.) fruit widely consumed fresh and in beverage as juice or wine has been extensively used in traditional medicine in various parts of the world. Pomegranate fruit is a rich source of many phenolic compounds, which include flavonoids (such as anthocyanins, catechins and other complex
flavanoids) and hydrolyzable tannins (such as punicalin, pendunculagin, punicalagin, gallic and ellagic esters of glucose) (14). Extracts from different parts of this plant such as juice (15), seed (16) and peel (17) have been reported to exhibit strong antioxidant activity. Studies have shown that pomegranate juice possesses anti proliferative (18), antiatherogenic (19), anti inflammatory and anti tumorigenic (20,21) effects. These effects of pomegranate-derived products appear to be attributable to its free radical scavenging and antioxidant properties (22).

Recently, we have shown that pomegranate fruit extract (PFE) exerts antitumor-promoting effect in mouse skin (20), inhibits UVB-mediated activation of nuclear factor kappa B (14) and modulates UVA-mediated cell proliferation pathways in human epidermal keratinocytes (23). In the present study, we evaluated the effect of a standardized preparation of PFE termed POMx against UVB-mediated markers of oxidative stress and photoaging in immortalized human keratinocytes HaCaT. Specifically, we determined the effect of POMx on UVB-induced changes in intracellular reduced glutathione (GSH) content, lipid peroxidation (LPO), expression of MMPs, tissue inhibitor of MMP-1 (TIMP-1) and c-fos, phosphorylation of MAPKs and c-jun.

MATERIALS AND METHODS

Materials. Phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK1/2 (Thr183/Tyr185) and phospho-p38 (Thr180/Tyr182) antibodies were purchased from Cell Signalling Technology (Beverly, MA); phospho-c-jun (Ser73) from Upstate Cell Signaling Solutions (Charlottesville, VA); MMP-1, MMP-2 and MMP-9 antibodies from Lab Vision Corporation (Fremont, CA); MMP-7 and TIMP-1 antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); antine or antirabbit secondary antibody horseradish peroxidase conjugate and ECL western blotting detection reagent was purchased from Amersham Life Science (Arlington Height, IL). Protease inhibitor cocktail Set III was from Calbiochem (La Jolla, CA); Novex precast tris-glycine gels were from Novex (Invitrogen, Carlsbad, CA); Dulbecco’s modified eagle’s media (DMEM), culture media were from Cambrex Biosciences (Walkersville, MD); fetal bovine serum from ATCC (Manassas, VA); GSH and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) from Sigma Chemicals (St. Louis, MO) and bicuschinonic acid (BCA) protein assay kit was obtained from Pierce Biotechnology Inc. (Rockford, IL). All other chemicals used were at least of analytical grade. POMx was provided by POM Wonderful, Inc. (Los Angeles, CA). Pomegranate fruit was sliced and squeezed for the juice and the remaining material including squeezed arils, rind and other parts were processed to remove the seeds before undergoing a series of concentration steps to produce a polyphenol rich POMx. POMx is a 70 Brix commercial grade of pomegranate extract concentrate with a polyphenol content of 135 000 p.p.m. gallic acid equivalent and ellagitannins as its major constituent.

Cell culture. The immortalized human keratinocytes, HaCaT, were cultured in DMEM containing 4.5 g L⁻¹ glucose and l-glutamine supplemented with 10% fetal bovine serum, 100 μg mL⁻¹ penicillin and 100 U mL⁻¹ streptomycin. The cells were maintained under standard cell culture conditions at 37°C and 5% CO₂ in a humid environment.

Treatment of cells. HaCaT cells were maintained in DMEM culture media up to a confluence of 40–60% and then pretreated with indicated concentrations of POMx in dimethyl sulfoxide (DMSO) (final concentration of DMSO used was 0.1% [vol/vol] for each treatment and control) for 24 h in DMEM medium after which the media were removed and cells were washed with phosphate-buffered saline (PBS). Fresh PBS was added and cells were irradiated with UVB using a custom designed Research Irradiation Unit (Daavlin, Bryan, OH) that consists of a fixture mounted on fixed legs. Mounted within the exposure unit are four UVB lamps and the exposure system is controlled using Daavlin Flex Control Integrating Dosimeters. In this system dose units can be entered in mJ cm⁻² for UVB and variations in energy output are automatically compensated to deliver the desired dose. Using this system, the cells were exposed to accurate dosimetry of UVB radiation. Cells were exposed to UVB 15 or 30 mJ cm⁻², after which PBS was removed, fresh media added. Cells were harvested 1 h after UVB irradiation for MAPKs, 6 h post UVB for phospho-c-jun, c-fos, MMPs, LPO and GSH. For trolox equivalent antioxidant capacity (TEAC), cells were harvested 24 h after POMx treatment without UVB exposure.

Cell growth/cell viability assay. Viability of cells in culture was measured by quantification of mitochondrial dehydrogenase activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly HaCaT cells were plated at 2 × 10⁴ cells per well in 200 μL of DMEM medium in a 96-well microtiter plate and cultured overnight followed by treatment with POMx, with or without UVB treatment at a dose of 15 and 30 mJ cm⁻² as detailed above. After culturing for another 24 h, cells were incubated with 100 μL of fresh media containing 0.1 mg mL⁻¹ MTT for 2 h at 37°C. The MTT solution was discarded from the wells by aspiration. Formazan crystals formed were dissolved in 100 μL of DMSO, and the absorbance was measured at 570 nm using a microplate reader (Thermobal Systems, Finland). The effect of POMx on growth inhibition was assessed as percentage cell viability where vehicle-treated cells were taken as 100% viability.

Trolox equivalent antioxidant capacity. The total antioxidant activity of POMx was measured by the trolox equivalent antioxidant assay, which measures the combined antioxidant activity of all the constituents using antioxidant assay kit (Cayman Chemicals Company, Ann Arbor, MI), following the manufacturer’s protocol. The assay relies on the ability of antioxidants to inhibit oxidation of ABTS (2,2’-Azino-di-[3-ethylbenzthiazoline sulphonate) to ABTS⁺ by metmyoglobin. The ABTS present is measured by reading its absorbance at 750 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E, was used as an antioxidant standard. Briefly cells were collected by scraping in ice cold buffer (5 mM potassium phosphate [pH 7.4] containing 0.9% sodium chloride and 1% glucose), lysed by sonication and centrifuged at 13 000 g for 15 min at 4°C. The supernatant was removed and stored on ice for the assay. Ten microliters of standard or sample was added to each well of 96-well plates followed by 10 μL of metmyoglobin and 150 μL of ABTS, and the reaction was initiated by addition of 40 μL of 441 μM hydrogen peroxide. The plate was incubated on a shaker at room temperature for 5 min and the absorbance read at 750 nm. The antioxidant capacity of samples was calculated from the standard calibration curve of trolox in terms of trolox equivalent per mg protein. Protein estimation was made by BCA method.

Reduced glutathione content. Glutathione content was measured spectrophotometrically after reacting with DTNB (24). The method is based on the ability of the thiol group to reduce DTNB and form a yellow-colored anionic product, the OD of which is measured at 412 nm. Briefly HaCaT cells were collected in cold 50 mM MES buffer (pH 6.0) containing 1 mM EDTA, sonicated on ice and centrifuged at 13 000 g for 15 min at 4°C. The supernatant was deproteinized by equal amount of 20% metaphosphoric acid, centrifuged and clear supernatant was used for the assay. Concentration of GSH MTE was determined from a standard plot as GSH per mg protein. Protein estimation was made by the BCA method.

Lipid peroxidation assay. Lipid peroxidation was assayed as thiobarbituric acid reacting substances (TBARS) in terms of malondialdehyde (MDA) equivalents by OXI-TEK (TBARS) assay kit (Alexis Biochemicals, Buffalo, NY) following the manufacturer’s protocol. Briefly, cells were collected in PBS and homogenized by sonication on ice and the whole cell homogenate was used for estimation of LPO. MDA standard or sample homogenate 100 μL was added to 100 μL of SDS solution, to it 2.5 mL of thiobarbituric acid-buffer reagent was added and incubated at 95°C for 60 min, cooled to room temperature on ice and centrifuged at 3000 g for 15 min and the absorbance of supernatant read at 532 nm. The extent of LPO was calculated from the standard calibration curve of MDA as MDA per mg protein. Protein estimation was made by the BCA method.

Whole cell lystate preparation. HaCaT cell lysates for western blot analysis were prepared by keeping the cells in 0.3 mL of lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1 mM EGTA) containing 0.2 mM sodium vanadate, 2 mM PMSF, 0.5% NP-40 and 0.2 μM aprotinin with freshly added
protease inhibitor cocktail at 4°C for 15 min. The cells were scraped and collected in a microfuge tube and then passed through a 22-1/2-G needle to break up the cell aggregates. The lysates were centrifuged at 13,000 g for 25 min at 4°C to remove cell debris. Supernatant was collected, and protein estimation was made by the BCA method.

SDS-polyacrylamide gel electrophoresis and western blot analysis. For western blot analysis, equal amount (30–40 µg) of protein was resolved electrophoretically over 12% tris glycine gel, and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked in blocking buffer (5% nonfat dry milk in 20 mm Tris-buffered saline, pH 7.6 containing 1% Tween 20 [TBST]) for 1 h at room temperature followed by incubation with appropriate primary antibody in blocking buffer for 2 h to overnight at 4°C. This was followed by incubation with specific antimouse or antirabbit secondary antibody horseradish peroxidase for 2 h at room temperature and then washed thrice, 15 min each in TBST and detected by enhanced chemiluminescence and autoradiography using Blue Lite Autorad film obtained from ISC Bioexpress (Kaysville, UT).

Statistical analysis. The results are expressed as mean plus minus standard deviation. Statistical analysis of data was performed using Student’s t-test and *P < 0.001 was considered statistically significant.

RESULTS

POMx protects HaCaT cells from UVB-mediated cytotoxicity

We first determined the effect of POMx (2.5–40 µg mL⁻¹) against UVB-mediated cytotoxicity by measuring cell viability. Irradiation of HaCaT cells with UVB (15–30 mJ cm⁻²) resulted in a significant decrease in cell viability. Data in Fig. 1 show that UVB-mediated cytotoxicity was significantly lower in cells pretreated with POMx (10–40 µg mL⁻¹) in a dose-dependent manner. POMx treatment to non-UVB-irradiated cells showed no cytotoxic effects at the doses studied.

POMx exerts strong antioxidant effect

In order to determine whether the observed protective effect of POMx against UVB-mediated decrease in cell viability is due to its antioxidant activity, we performed the TEAC assay. TEAC provides a tool for monitoring total antioxidant activity. HaCaT cells were pretreated with POMx (10–40 µg mL⁻¹) for 24 h and cell lysates were prepared as described. The doses of POMx (10–40 µg mL⁻¹) selected were based on the data shown in Fig. 1, as these doses provide significant protection against UVB-mediated cytotoxicity. We found that POMx exerted strong antioxidant activity, as TEAC increased in cells treated with POMx in a dose-dependent manner when compared with untreated cells (Fig. 2).

POMx inhibits UVB-mediated decrease in GSH levels in HaCaT cells

Among the cutaneous antioxidants, the tripeptide GSH (γ-glutamyl-cysteinyl-glycine) plays an important role in protecting skin cells from oxidative damage by directly scavenging ROS or acting as a coenzyme in GSH peroxidase or GSH-S-transferase catalyzed reactions. UVB-induced damage is mediated by generation of ROS resulting in oxidative stress that in turn results in depletion of endogenous antioxidants. We assessed the intracellular GSH levels in HaCaT cells, and found that treatment of cells with POMx resulted in a significant increase in intracellular GSH levels. UVB (15–30 mJ cm⁻²) irradiation resulted in a dose-dependent decrease in intracellular GSH. Pretreatment of cells with POMx (10–40 µg mL⁻¹) prior to UVB exposure inhibited UVB-mediated decrease in GSH contents (Fig. 3a).

POMx inhibits UVB-mediated increase in LPO in HaCaT cells

We then evaluated the effect of POMx on UVB-mediated increase in LPO, which is a well-accepted marker of oxidative stress. LPO in biological membranes is a free radical-mediated event and is regulated by the availability of substrates in the form of polyunsaturated fatty acids and prooxidants that promote peroxidation. LPO is highly detrimental to cell
membrane structure and function, and its elevated level has been linked to damaging effects such as loss of fluidity, inactivation of membrane enzymes, increased cell membrane permeability and ultimately ruptured cell membranes leading to release of cell organelles. As shown in Fig. 3b, UVB irradiation of HaCaT cells resulted in a significant induction of LPO (measured in terms of malondialdehyde equivalent) by 89% and 146% at UVB doses of 15 and 30 mJ cm$^{-2}$, respectively, when compared with untreated cells. Pretreatment of cells with POMx (10–40 µg mL$^{-1}$) was found to significantly inhibit UVB-mediated increase in LPO (Fig. 3b). Thus, inhibition of LPO by POMx would result in the reduction of risk factors associated with UVB radiation.

POMx inhibits UVB-mediated increase in MMP protein expression in HaCaT cell

Exposure to UVB radiation is known to upregulate the synthesis of matrix degrading enzymes, MMPs. MMPs are a family of structurally related zinc-dependent endopeptidases, which are capable of degrading a wide variety of ECM components and play an important role in photoaging. We therefore evaluated the effect of POMx on UVB-induced MMP expression in HaCaT cells. UVB (30 mJ cm$^{-2}$) irradiation of HaCaT cells resulted in a marked increase in MMPs-1, -2, -7 and -9 proteins (Fig. 4). Our data show that pretreatment of HaCaT cells with POMx (10–40 µg mL$^{-1}$) inhibited the UVB-mediated increase in MMPs-1, -2, -7 and -9 protein levels.

POMx inhibits UVB-mediated decrease in TIMP-1 protein expression in HaCaT cell

Extracellular matrix metabolism is tightly controlled by MMPs and their tissue inhibitors, TIMPs, and the balance between these plays an important role in the maintenance of tissue homeostasis. We therefore studied the effect of POMx on UVB-mediated altered TIMP-1 expression. Our data show that UVB (30 mJ cm$^{-2}$) caused a decrease in TIMP-1, whereas treatment of HaCaT cells with POMx (10–40 µg mL$^{-1}$) prior to UVB exposure inhibited UVB-mediated decrease of TIMP-1 protein (Fig. 4). These results show that POMx restores UVB-induced inhibition of TIMP-1 expression.

POMx inhibits UVB-induced phosphorylation of c-jun in HaCaT cells

Activator protein-1 (AP-1) is closely related to matrix degrading enzymes that induce breakdown of collagen. Jun proteins form homodimers or heterodimers with fos proteins to form AP-1 complexes. The transcriptional activity of AP-1 is dependent on the degree of phosphorylation of c-jun and expression of c-fos. We investigated the effect of POMx on UVB-induced phosphorylation of c-jun protein and expression of c-fos protein. Our results show that UVB (30 mJ cm$^{-2}$) irradiation of HaCaT cells increased the level of phosphorylated c-jun and c-fos proteins. Pretreatment of HaCaT cells with POMx (10–40 µg mL$^{-1}$) inhibited UVB-mediated phosphorylation of c-jun (Fig. 5). POMx did not cause any significant change against UVB-induced expression of c-fos protein.
POMx inhibits UVB-mediated phosphorylation of MAPKs in HaCaT cells

MAPKs encompass a large number of serine/threonine kinases involved in the regulation of a wide array of cellular processes including proliferation, differentiation, stress adaptation and apoptosis. The MAPKs are divided into three multimer subfamilies—extracellular signal regulated kinase (ERK-1/2), c-jun amino-terminal kinase (JNK-1/2) and p38. UVB-induced phosphorylations of MAPKs have been shown to be inhibited by the use of antioxidants, thereby suggesting that MAPKs are important targets affected by oxidative stress. We therefore investigated the effects of POMx on UVB-induced MAPKs in HaCaT cells using phospho-specific MAPKs antibodies. Our results show that UVB (30 mJ cm⁻²) irradiation induced the phosphorylation of MAPKs, whereas treatment of HaCaT cells with POMx (10–40 µg mL⁻¹) prior to UVB irradiation inhibited UVB-mediated phosphorylation of these MAPKs (Fig. 6).

DISCUSSION

Exposure of skin to UVB radiation results in increased ROS generation, which contributes to several pathological conditions, including photoaging and photocarcinogenesis (25,26). As ROS are implicated in skin damage by UVB, scavenging of these reactive species could prevent the oxidative reactions and subsequently protect skin from the damaging effects of UVB radiation. Therefore, the use of botanical antioxidants to reduce harmful effect of UV radiation by scavenging ROS is a novel approach to delay the process of photoaging. One such natural product is pomegranate, which is widely consumed in fresh and beverage forms and has been used extensively in ancient cultures for various medicinal properties (27). Pomegranate juice and extracts have potent antioxidant and antiproliferative properties (28). Studies have shown that pomegranate and other naturally occurring antioxidant-rich botanicals are effective in reducing the harmful effect of UVB-mediated skin damage (3,14,29).

Our result shows that the percentage of viable cells was markedly reduced after UVB-irradiation as compared to control cells. Cellular antioxidant mechanisms may be overwhelmed by excessive free radical generation altering the redox status of cell and affects cell viability. POMx protects the cells from UVB-mediated cell death (Fig. 1) by its strong antioxidant activity (Fig. 2), and therefore plays a significant role in ameliorating or preventing photo-biological damage.

Glutathione is an important intracellular nonprotein sulphydryl peptide with multiple functions ranging from antioxidant defense to modulation of cell proliferation. GSH plays a central role in the maintenance of cellular redox homeostasis, regulating signaling pathways modulated by oxidative stress and protects skin cell against oxidative injury (30). UVB-mediated loss of cell viability is associated with a marked decrease in GSH content, indicating an impairment of the antioxidant pool, causing an increase in ROS, and may predispose the cell to a lower defense against condition of oxidative stress. GSH depletion was significantly and dose-dependently inhibited by pretreatment of cells with POMx (Fig. 3a). POMx due to its high antioxidant activity protects cells from UVB-induced oxidative stress by increasing their antioxidative status. Other antioxidants such as ascorbic acid have also been reported to protect skin cells against UVB-induced GSH depletion (31).

Oxidative damage to lipids and proteins, an immediate consequence of UV radiation to skin, occurs most readily in the superficial layers (32). Sustained oxidative insult as a result of UVB exposure causes LPO, which leads to the accumulation of MDA, a stable end product of LPO, indirectly suggesting the generation of ROS. Increased LPO caused by UVB irradiation may evoke immune and inflammatory responses and activate gene expression resulting from membrane-dependent oxidative damage (2). POMx significantly reduced peroxide accumulation, suggesting that POMx due to its antioxidant activity could scavenge ROS and inhibit the reaction of LPO (Fig. 3b).

UVB radiation has been reported to cause induction of different MMPs that degrade collagen. MMP-mediated collagen damage has been shown to be a major contributor of photoaged human skin. Although irradiation-induced expression of MMP gene occurs predominantly in the epidermis, MMP proteins and their enzymatic activity are abundant in both the dermis and the epidermis (33,34). UVB irradiation
which induces secretion of MMPs in skin cells, such as keratinocytes, fibroblasts and inflammatory cells, contribute substantially to the connective tissue damage that occurs during photoaging. Both MMPs and TIMPs are primary enzymes regulating the metabolism of collagen, but their effects are opposite. MMPs decompose the ECM while TIMPs inhibit the activity of MMPs and prevent breakdown of the ECM (35). TIMP-1 is important in ECM metabolism as it can inhibit the activity of all MMPs except MT-MMP (36). Our results demonstrate that UVB-irradiation induced MMP-1, MMP-2, MMP-7 and MMP-9 in HaCaT keratinocytes whereas it inhibited TIMP-1 protein expression (Fig. 4). Pretreatment of HaCaT cells with POMx abrogated this effect. Therefore, inhibition of the induction of MMPs can alleviate UV-induced photoaging in terms of protection from collagen destruction.

Recent studies suggest that UV radiation-induced generation of ROS contributes significantly to signaling events, leading to gene expression (37). The c-jun gene which encodes the nuclear phosphoprotein c-jun, in association with c-fos, binds to the AP-1 sites of DNA and acts as a regulatory factor for gene transcription (38). It has been proposed that activation of AP-1 participates in the UV-driven breakdown of the dermal ECM in human skin by inducing the expression of a series of metalloproteinases responsible for collagen degradation (39). Our results show that UVB irradiation of HaCaT cells increased the level of phosphorylated c-jun along with c-fos whereas pretreatment of HaCaT keratinocytes with POMx inhibited UVB-induced phosphorylation of c-jun (Fig. 5). These results show that inhibition of c-jun phosphorylation, which is known to be closely associated with UV-induced AP-1 activation, likely contributes to the prevention of UVB-induced AP-1-regulated MMPs in human skin.

UVB-induced phosphorylation of MAPK proteins, namely ERK1/2, JNK1/2 and p38, has been implicated in various skin diseases, including skin cancer and photoaging. The phosphorylation of the MAPK proteins is known to be mediated through UVB-induced oxidative stress (40). UVB-induced phosphorylation of MAPKs has been shown to be inhibited by the use of antioxidants, thereby suggesting that these are important targets affected by ROS (41). Accumulating evidence indicates that UV radiation also activates cell surface growth factor and cytokine receptors which stimulate MAPK signal transduction pathways (42,43). Our data demonstrate that pretreatment of HaCaT cells with POMx inhibited UVB-mediated phosphorylation of ERK1/2, JNK1/2 and p38 (Fig. 6). As MAPKs are related to c-jun phosphorylation and expression, with subsequent AP-1 activation (44), the inhibition of UVB-induced c-jun phosphorylation by these pomegranate-derived products may be mediated by inhibition of phosphorylation of MAPKs.

In conclusion, we have demonstrated the antioxidative and photochemopreventive effect of POMx. Our data suggest that pretreatment of HaCaT cells with POMs inhibited UVB-mediated decrease in cell viability, decrease in intracellular GSH content and increase in LPO. POMs attenuated UVB-induced phosphorylation of MAPKs and c-jun protein. These results provide a basis for more in-depth studies to assess the effectiveness of pomegranate fruit and its derived products in the prevention of UVB-mediated damage and photoaging.

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