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Pomegranate Fruit Extract Modulates UV-B–mediated Phosphorylation of Mitogen-activated Protein Kinases and Activation of Nuclear Factor Kappa B in Normal Human Epidermal Keratinocytes¶

Farrukh Afaq[1], Arshi Malik[1], Deeba Syed[1], Daniel Maes[2], Mary S. Matsui[2] and Hasan Mukhtar*[1]

[1]Department of Dermatology, University of Wisconsin, Madison, WI
[2]Biological Research Department, Estee Lauder Companies Inc., Melville, NY

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ABSTRACT

Excessive exposure of solar ultraviolet (UV) radiation, particularly its UV-B component, to humans causes many adverse effects that include erythema, hyperplasia, hyperpigmentation, immunosuppression, photoaging and skin cancer. In recent years, there is increasing use of botanical agents in skin care products. Pomegranate derived from the tree *Punica granatum* contains anthocyanins (such as delphinidin, cyanidin and pelargonidin) and hydrolyzable tannins (such as punicalin, pedunculagin, punicalagin, gallagic and ellagic acid esters of glucose) and possesses strong antioxidant and anti-inflammatory properties. Recently, we have shown that pomegranate fruit extract (PFE) possesses antitumor promoting effects in a mouse model of chemical carcinogenesis. To begin to establish the effect of PFE for humans in this study, we determined its effect on UV-B–induced adverse effects in normal human epidermal keratinocytes (NHEK). We first assessed the effect of PFE on UV-B–mediated phosphorylation of mitogen-activated protein kinases (MAPK) pathway in NHEK. Immunoblot analysis demonstrated that the treatment of NHEK with PFE (10–40 μg/mL) for 24 h before UV-B (40 mJ/cm^2) exposure dose dependently inhibited UV-B–mediated phosphorylation of ERK1/2, JNK1/2 and p38 protein. We also observed that PFE (20 μg/mL) inhibited UV-B–mediated phosphorylation of MAPK in a time-dependent manner. Furthermore, in dose- and time-dependent studies, we evaluated the effect of PFE on UV-B–mediated activation of nuclear factor kappa B (NF-κB) pathway. Using Western blot analysis, we found that PFE treatment of NHEK resulted in a dose- and time-dependent inhibition of UV-B–mediated degradation and phosphorylation of IκBα and activation of IKKα. Using immunoblot analysis, enzyme-linked immunosorbent assay and electrophoretic mobility shift assay, we found that PFE treatment to NHEK resulted in a dose- and time-dependent inhibition of UV-B–mediated nuclear translocation and phosphorylation of NF-κB/p65 at Ser^{536}. Taken together, our data shows that PFE protects against the adverse effects of UV-B radiation by inhibiting UV-B–induced modulations of NF-κB and MAPK pathways and provides a molecular basis for the photochemopreventive effects of PFE.

INTRODUCTION

Excessive exposure of solar ultraviolet (UV) radiation, particularly its UV-B component, to humans causes many adverse effects that include erythema, hyperplasia, hyperpigmentation, immunosuppression, photoaging and skin cancer (1–3). Among all adverse effects of UV-B, skin cancer is of greatest concern because the incidence of nonmelanoma skin cancer has been increasing at an alarming rate and is the leading cause of malignancy in the United States (1–4). UV-B radiation to mammalian skin directly or indirectly targets the DNA by producing cyclobutane pyrimidine dimers, pyrimidine–pyrimidone (6–4) photoproducts and oxidative DNA base damage (5,6), generation of reactive oxygen species (ROS) (7,8), oxidative stress (9,10) and the resultant alterations in a variety of signaling events (2,8,11). UV-B–mediated activation of mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NF-κB) plays an important role in inflammation, immunity, cell proliferation and skin carcinogenesis (2,8,11).

Primary prevention of skin cancer, which includes the use of sunscreens or wearing of protective clothing, is recommended for reducing the risk of skin cancer but for many reasons these primary prevention approaches have met with limited success (1,2). Photochemoprevention strategies could be combined with primary preventive strategies for more effective prevention of skin cancer. The endogenous antioxidant capacity of the skin is a major determinant in its response to UV-induced oxidative stress–mediated skin

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*To whom correspondence should be addressed: Department of Dermatology, Medical Sciences Centre, Room No. B25, 1300 University Avenue, Madison, WI 53706, USA. Fax: 608-263-5223; e-mail: hmukhtar@wisc.edu

Abbreviations: DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(aminoethylether)-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IKK, IκB kinases; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor kappa B; NHEK, normal human epidermal keratinocytes; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PFE, pomegranate fruit extract; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; TdT, terminal deoxynucleotidyl transferase; UV, ultraviolet.

damage. Skin possesses an elaborate antioxidant defense system but because of constant environmental exposure to physical and chemical agents, an oxidant–antioxidant imbalance leading to overwhelming generation of ROS results, which plays a major role in all stages of skin cancer development. Regular intake of dietary antioxidants or treatment of the skin with products containing antioxidant ingredients may be a useful strategy for the prevention of UV-mediated cutaneous damages (1,11–13). This has sparked the use of exogenous supplementation of antioxidants, notably of botanical origin in skin care products.

Pomegranate (*Punica granatum*, Punicaceae) believed to date back to the Garden of Eden has been used in folk medicine for centuries. The edible fruit is native to Persia and is cultivated in Mediterranean countries, Afghanistan, India, China, Japan, Russia and the United States. Edible parts of pomegranate fruit (about 80% of total fruit weight) comprise 80% juice and 20% seed. Pomegranate fruit extract (PFE) is a rich source of anthocyanins (such as delphinidin, cyanidin and pelargonidin) and hydrolyzable tannins (such as punicalin, pedunculagin, punicalagin, gallagic and ellagic acid esters of glucose) (14). The hydrolyzable tannins account for 92% of the antioxidant activity of the whole fruit (15). Studies have shown that PFE possesses strong antioxidant (15,16), anti-inflammatory (14), antiproliferative (17), antiatherogenic (18) and antitumorigenic properties (14). Recently, we have demonstrated that topical application of PFE prevents 7,12-dimethylbenz(a)anthacene–initiated and 12-*O*-tetradecanoylphorbol-13-acetate–promoted skin carcinogenesis in CD-1 mice via modulations in MAPK and NF-κB pathways (14). For relevance of this study to human skin, we determined the photochemopreventive potential of PFE in an *in vitro* situation by studying its effect on UV-B–mediated activation of MAPK and NF-κB.

MATERIALS AND METHODS

Materials. ERK1/2 (phospho-p44/42, Th202/Tyr204), JNK1/2 (phospho-p54/46, Th183/Tyr185), p38 (phospho-p38, Th180/Tyr204) and phospho NF-κB antibodies were purchased from Cell Signaling Technology (Beverly, MA). IκBα and IκBα (phospho) antibodies were obtained from New England Biolabs Inc. (Beverly, MA). NF-κB/p65 antibody was procured from Geneka Biotechnology Inc. (Montreal, Canada). IKKα antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate was obtained from Amersham Life Science Inc. (Arlington Height, IL). Trans-AM NF-κB/p65 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Active Motif North America (Carlsbad, CA). Lightshift™ chemiluminiscent electrophoretic mobility shift assay (EMSA) kit was obtained from Pierce (Rockford, IL). The DC BioRad Protein assay kit was purchased from BioRad Laboratories (Hercules, CA). Novex precast Tris–glycine gels were obtained from Invitrogen (Carlsbad, CA). A commercial preparation of PFE provided by the Estee Lauder Companies Inc. (Melville, NY) was used in this study. In our recent publication, we have analyzed the PFE using a novel technique of matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The preparation of PFE was found to contain two types of polyphenolic compounds; anthocyanins (such as delphinidin, cyanidin and pelargonidin) and hydrolyzable tannins (such as punicalin, pedunculagin, punicalagin, gallagic and ellagic acid esters of glucose) (14).

Cell culture. The normal human epidermal keratinocytes (NHEK) were obtained from Invitrogen Corporation, and the primary cultures were maintained in keratinocyte-SFM (serum free medium) (Life Technologies, Grand Island, NY) supplemented with 0.1 m*M* calcium, 0.2% (vol/vol) bovine pituitary extract, epidermal growth factor (EGF) (10 ng/mL), insulin (5 μg/mL), hydrocortisone (5×10^{-7} *M*), ethanolamine (1×10^{-4} *M*), phosphoethanolamine (1×10^{-4} *M*) and L-glutamine. The cells were maintained at 95% humidity in 5% CO_2 environment at 37°C. The cells between third and fifth passage were used in this study.

Treatment of cells. PFE dissolved in dimethyl sulfoxide (DMSO) was used for the treatment of cells. The final concentration of DMSO used was 0.1% (vol/vol) for each treatment. For dose-dependent studies, the cells (70–80% confluent) were treated with PFE (10, 20, 40 μg/mL) for 24 h in keratinocyte-SFM, after which the media was removed and cells were washed with phosphate-buffered saline (PBS), then fresh PBS was added and these PFE pretreated cells were exposed to UV-B (40 mJ/cm^2). For UV-B exposure, the cells were irradiated with 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 UV-B lamps and the exposure system is controlled using Daavlin Flex Control Integrating Dosimeters. In this system, dose units can be entered in millijoules per square centimeter for UV-B. This exposure unit is currently used in our laboratory, which enables us to enter dose in millijoules and variations in energy output are automatically compensated so the desired dose is delivered. Using this system, the cells were exposed to accurate dosimetery of UV-B radiation. One hour after UV-B exposure (for MAPK studies) and 6 h after UV-B exposure (for NF-κB studies), the cells were harvested and total cell, cytosolic and nuclear lysates were prepared as described below. For time-dependent studies, the cells (60–70% confluent) were treated with PFE (20 μg/mL) for 24 h, after which the media was removed and cells were washed with PBS, then fresh PBS was added and these PFE-pretreated cells were exposed to UV-B (40 mJ/cm^2). At different time points after UV-B exposure (15 and 30 min, 1, 2, 3, 6, 12 h), cells were harvested and total cell, cytosolic and nuclear lysates were prepared.

Preparation of total cell lysate. After treatment of cells with PFE or UV-B (or both), the medium was aspirated and the cells were washed twice in PBS (10 m*M*, pH 7.4). The cells were incubated in 0.4 mL ice-cold lysis buffer (50 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* ethyleneglycol-bis (aminoethylether)-tetraacetic acid [EGTA], 1 m*M* ethylenediaminetetraacetic acid [EDTA], 20 m*M* NaF, 100 m*M* Na_3VO_4, 0.5% Nonidet P-40 [NP-40], 1% Triton X-100, 1 m*M* phenylmethylsulfonyl fluoride [PMSF] [pH 7.4]) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA). The cells were then centrifuged at 14 000 ***g*** for 25 min at 4°C, and the supernatant (total cell lysate) was collected, aliquoted and stored at –80°C.

Preparation of cytosolic and nuclear lysates. After treatment of cells with PFE or UV-B (or both), the medium was aspirated and the cells were washed twice in PBS (10 m*M*, pH 7.4). The cells were incubated in 0.4 mL ice-cold lysis buffer (10 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* dithiothreitol [DTT], 1 m*M* PMSF [pH 7.9]) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem) for 15 min, after which 12.5 μL of 10% NP-40 was added and the contents were mixed on a vortex and then centrifuged for 1 min (14 000 ***g***) at 4°C. The supernatant was saved as cytosolic lysate and stored at –80°C. The nuclear pellet was resuspended in 50 μL of ice-cold nuclear extraction buffer (20 m*M* HEPES, 0.4 *M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* DTT, 1 m*M* PMSF [pH 7.9]) with freshly added protease inhibitor cocktail for 30 min with intermittent mixing. The cells were centrifuged for 5 min (14 000 ***g***) at 4°C, and the supernatant (nuclear extract) was stored at –80°C. The protein concentration was determined by the DC BioRad assay using the manufacturer's protocol (BioRad Laboratories, Hercules, CA).

Enzyme-linked immunosorbent assay. The commercially available Trans-AM kit used for the assay of NF-κB/p65 uses an oligonucleotide containing NF-κB consensuses site (5′-GGGACTTTCC-3′) that binds to the nuclear extract and can detect NF-κB, which can recognize an epitope on p65 activated and bound to its target DNA. In the absence of the competitive binding with the wild-type or mutated consensus oligonucleotide, 30 μL of binding buffer was added to each well in duplicate. Alternatively, 30 μL of binding buffer containing 20 pmol (2 μL) of appropriate oligonucleotide, in duplicate, was added to the corresponding wells. Ten micrograms of protein from the nuclear lysate of each sample diluted in 20 μL lysis buffer was loaded per well. For positive control, 20 μL of lysis buffer containing 1 μL of control cell extract per well, and for blank, 20 μL of lysis buffer per well was used. The plate was sealed with the adhesive film and incubated for 1 h at room temperature with mild agitation (100 rpm on a rocking platform), after which the well was washed three times with 200 μL of 1× wash buffer and 100 μL of diluted primary antibody (1:1000 dilution in 1× antibody-binding buffer) was added to each well and incubated at room temperature for 1 h without agitation. The wells were washed again three times with 1× wash buffer and 100 μL of diluted horseradish peroxidase-conjugate antibody (1:1000 dilution in 1× antibody-binding buffer) was added to each well and incubated for 1 h. The wells were again washed four times with 1× wash buffer followed by the addition of 100 μL of developing solution. The content was incubated for 5 min at room temperature. This was followed by

an addition of 100 μL of stop solution to each well, and the absorbance was read within 5 min at 450 nm.

Electrophoretic mobility shift assay. EMSA for NF-κB was performed using lightshift™ chemiluminiscent EMSA kit (Pierce) by following the manufacturer's protocol. To start with, DNA was biotin labeled using the Biotin 3′ end labeling kit (Pierce). In brief, in a 50 μL reaction buffer, 5 pmol of double-stranded NF-κB oligonucleotide 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; 3′-TCA ACT CCC CTG AAA GGG TCC G-5′ was incubated in a microfuge tube with 10 μL of 5× terminal deoxynucleotidyl transferase (TdT) buffer, 5 μL of 5 μ*M* biotin-N4-CTP, 10 U of diluted TdT and 25 μL of ultrapure water at 37°C for 30 min. The reaction was stopped by the addition of 2.5 μL of 0.2 *M* EDTA. To labeled DNA, 50 μL of chloroform–isoamyl alcohol (24:1) was added to each tube and centrifuged briefly at 13 000 ***g***. The top aqueous phase containing the labeled DNA was removed and saved for binding reactions. Each binding reaction contained 1× binding buffer (100 m*M* Tris, 500 m*M* KCl, 10 m*M* DTT, pH 7.5), 2.5% glycerol, 5 m*M* $MgCl_2$, 50 ng/μL poly (dI-dC), 0.05% NP-40, 5 μg of nuclear extract and 20–50 fm of biotin end-labeled target DNA. The contents were incubated at room temperature for 20 min. To this reaction mixture was added 5 μL of 5× loading buffer, subjected to gel electrophoresis on a native polyacrylamide gel and transferred to a nylon membrane. When the transfer was complete, DNA was cross-linked to the membrane at 120 mJ/cm^2 using a UV cross-linker equipped with 254 nm bulbs. The biotin end-labeled DNA was detected using streptavidin–horseradish peroxidase conjugate and a chemiluminescent substrate. The membrane was exposed to X-ray film (XAR-5, Amersham Life Science) and developed using a Kodak film processor.

Western blot analysis. For Western blot analysis, 25–30 μg of protein was resolved over 8–12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked in blocking buffer (5% nonfat dry milk, 1% Tween 20 in 20 m*M* Tris-buffered saline, pH 7.6) for 1 h at room temperature followed by incubation with appropriate monoclonal primary antibody in blocking buffer for 1 h to overnight at 4°C. This was followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase (Amersham Life Sciences) for 1 h and then washed several times and detected by chemiluminescence (ECL kit, Amersham Life Sciences) and autoradiography using XAR-5 film obtained from Eastman Kodak Co. (Rochester, NY).

RESULTS

PFE treatment to NHEK inhibits UV-B–mediated phosphorylation of ERK1/2, JNK1/2 and p38

We and others have shown that UV-B irradiation resulted in an increased phosphorylation of MAPK in NHEK (8,19). To determine whether PFE could inhibit UV-B–mediated phosphorylation of MAPK (such as ERK1/2, JNK1/2 and p38) in NHEK, Western blot analysis was performed using phospho-specific MAPK antibodies. In the initial studies, we found that UV-B dose of 40 mJ/cm^2 significantly phosphorylates MAPK in NHEK 1 h after UV-B exposure (data not shown). Therefore, for dose–response study, we selected a time point of 1 h after UV-B exposure. We observed that pretreatment of NHEK with PFE (10, 20, 40 μg/mL) before UV-B irradiation inhibited UV-B–mediated phosphorylation of MAPK in a dose-dependent manner (Fig. 1A). In this study as evident from Western blot analysis, we found that UV-B exposure to NHEK resulted in the phosphorylation of ERK1/2 (p44 and p42) protein as early as 15 min and persisted till 2 h after UV-B exposure and thereafter a decrease in the phosphorylation of ERK1/2 protein was observed (Fig. 1B). Pretreatment of NHEK with PFE (20 μg/mL) before UV-B irradiation was found to inhibit UV-B–mediated phosphorylation of ERK1/2 in a time-dependent manner (Fig. 1B). We also found that UV-B irradiation resulted in an increased expression of the phosphorylated form of JNK1/2 (p54 and p46) and p38 as early as 30 min after UV-B exposure and persisted till 6 h after UV-B exposure. Pretreatment of NHEK with PFE (20 μg/mL) before UV-B irradiation markedly reduced UV-B–mediated phosphorylation of JNK1/2 and p38 in a time-dependent manner (Fig. 1B).

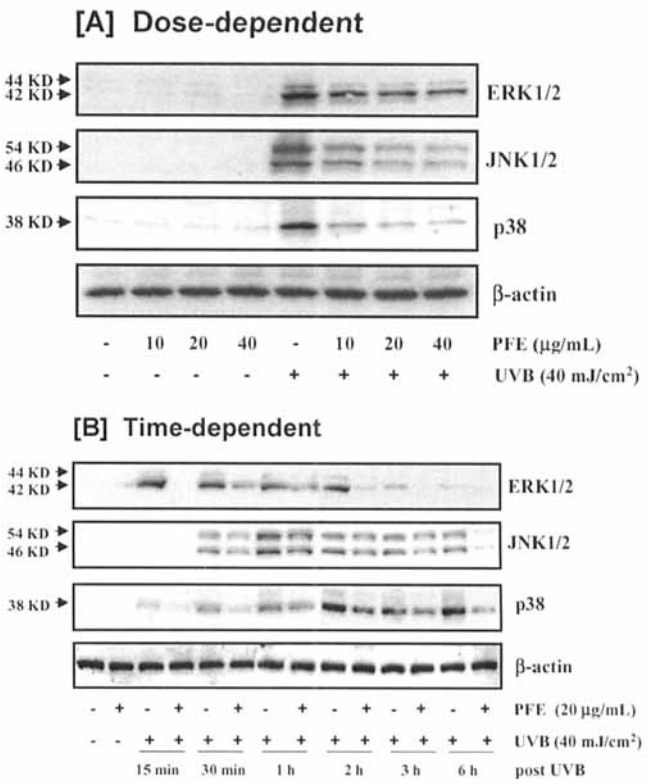

Figure 1. PFE treatment to NHEK inhibits UV-B–mediated phosphorylation of ERK1/2, JNK1/2 and p38. For dose-dependent studies, cells were pretreated with PFE (10, 20 and 40 μg/mL) for 24 h, after which the media was removed and cells were washed once with PBS, then fresh PBS was added and cells were exposed to UV-B (40 mJ/cm^2). One hour after UV-B exposure, the cells were harvested and total cell lysate was prepared (A). For time-dependent studies, cells were pretreated with PFE (20 μg/mL) for 24 h, after which the media was removed and cells were washed once with PBS, then fresh PBS was added and cells were exposed to UV-B (40 mJ/cm^2) (B). At different time points, cells were harvested and total cell lysate was prepared for Western blot analysis as detailed in Materials and Methods. The data shown here are from one representative experiment repeated two times with similar results.

PFE treatment to NHEK inhibits UV-B–mediated phosphorylation of NF-κB

Phosphorylation of NF-κB has been recently suggested as an additional step in the cascade of events leading to NF-κB activation (20,21). This phosphorylation is correlated with the binding of the nuclear NF-κB to the DNA and the formation of nuclear complexes with transcription factor coactivators or repressors. Studies have also shown tumor necrosis factor α induced phosphorylation of NF-κB/p65 at Ser^{536} in HeLa cells (22). In this study using an antibody that specifically recognizes Ser^{536}-phosphorylated p65, we demonstrated that NHEK irradiated with UV-B resulted in phosphorylation of p65 at Ser^{536} in the cytoplasm that was evident even at 15 min after irradiation and persisted till 12 h. As evident from Western blot analysis, we found that pretreatment of NHEK with PFE (10, 20, 40 μg/mL) resulted in a dose-dependent inhibition of UV-B–mediated phosphorylation of NF-κB/p65 at Ser^{536} (Fig. 2A). In a time-dependent study, we found that pre-

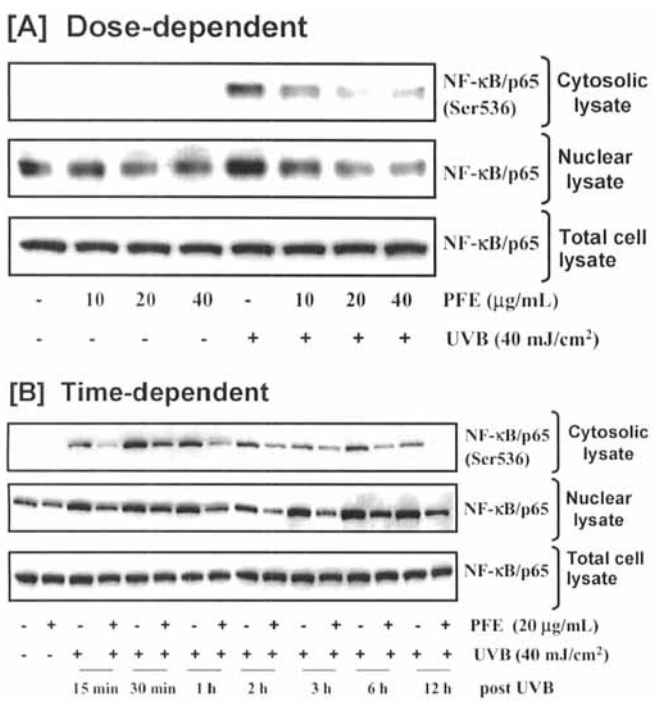

Figure 2. PFE treatment to NHEK inhibits UV-B–mediated phosphorylation and activation of NF-κB. For dose-dependent studies, cells were pretreated with PFE (10, 20 and 40 μg/mL) for 24 h, after which the media was removed and cells were washed once with PBS, then fresh PBS was added and cells were exposed to UV-B (40 mJ/cm^2). Six hours after UV-B exposure, the cells were harvested and total cell, cytosolic and nuclear lysates were prepared (A). For time-dependent studies, cells were pretreated with PFE (20 μg/mL) for 24 h, after which the media was removed and cells were washed once with PBS, then fresh PBS was added and cells were exposed to UV-B (40 mJ/cm^2) (B). At different time points, cells were harvested, total, cytosolic and nuclear lysates were prepared for Western blot analysis as detailed in Materials and Methods. The data shown here are from one representative experiment repeated two times with similar results.

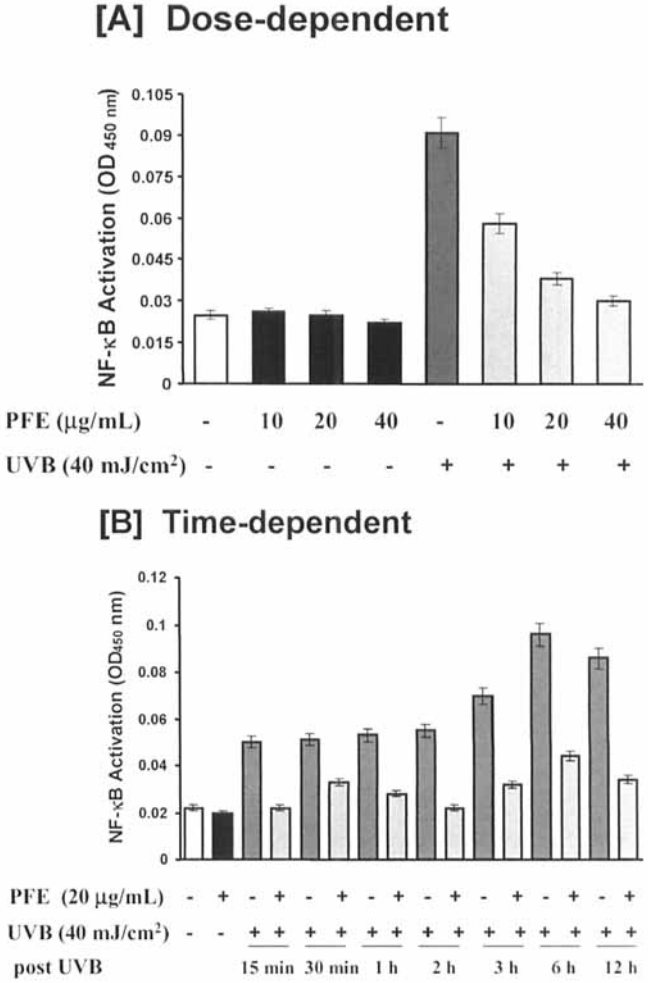

Figure 3. PFE treatment to NHEK inhibits UV-B–mediated activation and nuclear translocation of NF-κB. For dose-dependent studies, cells were pretreated with PFE (10, 20 and 40 μg/mL) for 24 h, after which the media was removed and cells were washed once with PBS, then fresh PBS was added and cells were exposed to UV-B (40 mJ/cm^2). Six hours after UV-B exposure, the cells were harvested and nuclear lysate was prepared (A). For time-dependent studies, cells were pretreated with PFE (20 μg/mL) for 24 h, after which the media was removed and cells were washed once with PBS, then fresh PBS was added and cells were exposed to UV-B (40 mJ/cm^2) (B). At different time points, cells were harvested and nuclear lysate was prepared for ELISA as detailed in Materials and Methods. The data shown here are from one representative experiment repeated three times with similar results.

treatment of NHEK with PFE (20 μg/mL) resulted in a time-dependent inhibition of UV-B–mediated phosphorylation of NF-κB/p65 (Fig. 2B).

PFE treatment to NHEK inhibits UV-B–mediated activation and nuclear translocation of NF-κB

Previous studies have shown that UV radiation results in an activation of NF-κB (11,23). We therefore determined whether pretreatment of NHEK with PFE inhibits UV-B–induced activation and nuclear translocation of NF-κB/p65. Western blot analysis data revealed that pretreatment of NHEK with PFE inhibited UV-B–mediated activation and nuclear translocation of NF-κB/p65 in a dose- and time-dependent manner (Fig. 2A,B). However, no change in the protein expression of NF-κB/p65 was observed after UV-B radiation in the total cell lysate (Fig. 2A,B). To further confirm our results, we also performed ELISA and found that pretreatment of NHEK with PFE (10, 20, 40 μg/mL) inhibited UV-B–induced activation and nuclear translocation of NF-κB/p65 in a dose-dependent manner (Fig. 3A). In a time-dependent study, we observed that UV-B–mediated activation of NF-κB/p65 started as early at 30 min after UV-B exposure, with maximum activation at 6 h after UV-B exposure and then persisted till 12 h after UV-B exposure. For time-dependent study, we used 20 μg/mL dose of PFE that was found to inhibit NF-κB/p65 activation and its nuclear translocation (Fig. 3B).

PFE treatment to NHEK inhibits UV-B–mediated NF-κB DNA-binding activity

Furthermore, we performed EMSA to investigate the effect of PFE treatment to NHEK on UV-B–mediated NF-κB DNA-binding activity. As shown in Fig. 4A, UV-B treatment resulted in a dramatic increase of NF-κB DNA-binding activity in comparison with control and PFE (10, 20, 40 μg/mL) alone–treated groups. Pretreatment of NHEK with PFE significantly inhibited UV-B–induced NF-κB DNA-binding activity in a dose-dependent manner (Fig. 4A). We also performed time-dependent study using a single

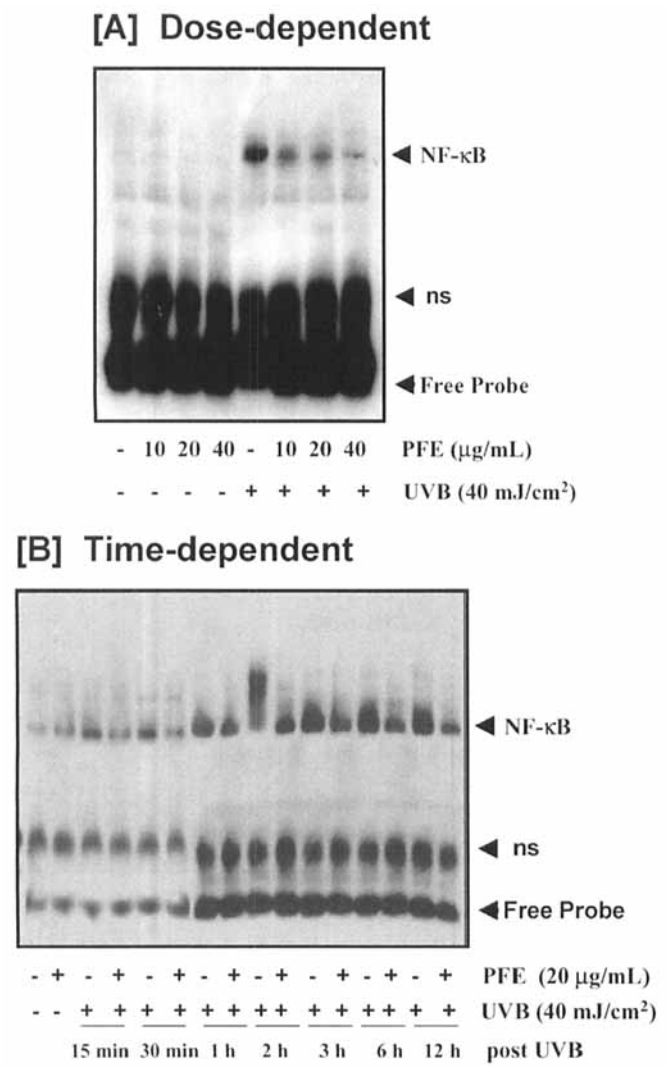

Figure 4. PFE treatment to NHEK inhibits UV-B–mediated NF-κB DNA-binding activity. For dose-dependent studies, cells were pretreated with PFE (10, 20 and 40 μg/mL) for 24 h, after which the media was removed and cells were washed once with PBS, then fresh PBS was added and cells were exposed to UV-B (40 mJ/cm^2). Six hours after UV-B exposure, the cells were harvested and nuclear lysate was prepared (A). For time-dependent studies, cells were pretreated with PFE (20 μg/mL) for 24 h, after which the media was removed and cells were washed once with PBS, then fresh PBS was added and cells were exposed to UV-B (40 mJ/cm^2) (B). At different time points, cells were harvested and nuclear lysate was prepared for EMSA as detailed in Materials and Methods. The data shown here are from one representative experiment repeated two times with similar results.

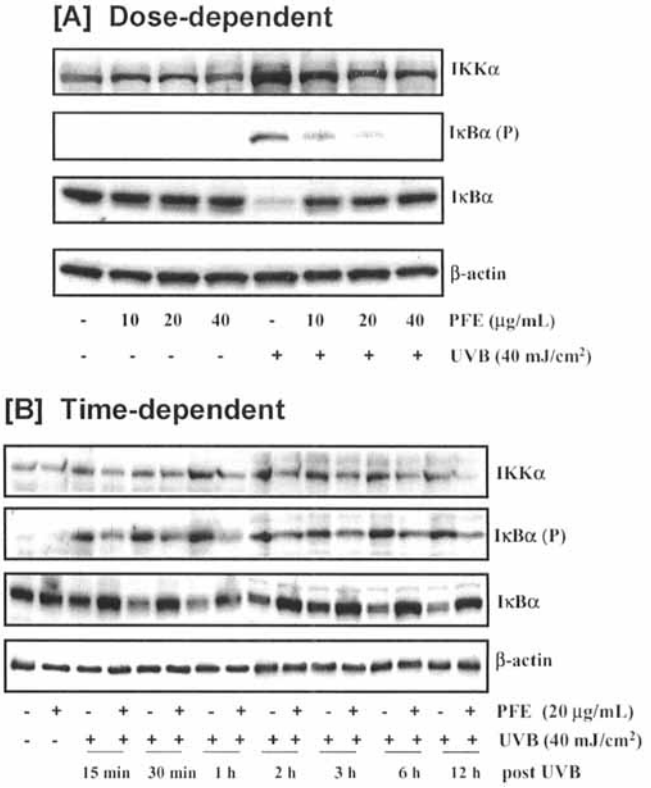

Figure 5. PFE treatment to NHEK inhibits UV-B–mediated activation of IKKα and phosphorylation and degradation of IκBα. For dose-dependent studies, cells were pretreated with PFE (10, 20 and 40 μg/mL) for 24 h, after which the media was removed and cells were washed once with PBS, then fresh PBS was added and cells were exposed to UV-B (40 mJ/cm^2). Six hours after UV-B exposure, the cells were harvested and cytosolic lysate was prepared (A). For time-dependent studies, cells were pretreated with PFE (20 μg/mL) for 24 h, after which the media was removed and cells were washed once with PBS, then fresh PBS was added and cells were exposed to UV-B (40 mJ/cm^2) (B). At different time points, cells were harvested and cytosolic lysate was prepared for Western blot analysis as detailed in Materials and Methods. The data shown here are from one representative experiment repeated three times with similar results.

dose of PFE (20 μg/mL). After 24 h of pretreatment, the cells were exposed to UV-B, and at different time points, post–UV-B exposure EMSA was performed. We observed that UV-B exposure resulted in an increased NF-κB DNA-binding activity in a time-dependent manner. Treatment of NHEK with PFE (20 μg/mL) resulted in a significant inhibition against UV-B–induced NF-κB DNA-binding activity (Fig. 4B).

PFE treatment to NHEK inhibits UV-B–mediated activation of IKKα and phosphorylation and degradation of IκBα

IKKα activity has been shown to be necessary for IκBα protein phosphorylation–degradation induced by UV-B radiation. To evaluate the possible inhibitory mechanism of PFE on IκBα protein degradation, we measured IKKα protein level. Western blot analysis showed that UV-B (40 mJ/cm^2) radiation resulted in the activation of IKKα in NHEK. Pretreatment of NHEK with PFE before UV-B inhibits UV-B–mediated activation of IKKα in a dose-dependent manner (Fig. 5A). For time-dependent study, we observed that 20 μg/mL dose of PFE was found to inhibit UV-B–induced activation of IKKα in a time-dependent manner (Fig. 5B). The translocation of NF-κB to the nucleus is preceded by the phosphorylation and the proteolytic degradation of IκBα. We next assessed whether UV-B exposure affects the phosphorylation of IκBα protein. As shown by Western blot, UV-B irradiation resulted in an increased phosphorylation of IκBα protein at Ser^{32} (Fig. 5A). In dose-dependent study, pretreatment of NHEK with PFE (10, 20, 40 μg/mL) resulted in a significant inhibition in UV-B–mediated increase in the phosphorylation of IκBα protein. In time-dependent study, we found that UV-B induced phosphorylation of IκBα protein 15 min after UV-B exposure and remained elevated till 12 h. Interestingly, we observed that pretreatment of NHEK with PFE (20 μg/mL) drastically decreased UV-B–induced phosphorylation of IκBα protein as evident by Western blot analysis (Fig. 5B). In this study, we also observed that UV-B exposure to NHEK resulted

in degradation of IκBα protein (Fig. 5A,B) and because of this degradation NF-κB/p65 is activated and translocated in to the nucleus (Figs. 2A,B and 3A,B). We were interested to see whether the degradation of IκBα is inhibited by PFE that will in turn inhibit the activation and translocation of NF-κB/p65. Western blot analysis revealed that treatment of NHEK with PFE (10, 20, 40 μg/mL) inhibits UV-B–mediated degradation of IκBα protein (Fig. 5A). In time-dependent study, we observed that pretreatment of NHEK with PFE (20 μg/mL) resulted in a significant inhibition against UV-B–induced degradation of IκBα protein as evident by Western blot analysis (Fig. 5B).

DISCUSSION

Solar UV radiation, particularly its UV-B component (290–320 nm), is believed to be the major cause of a variety of cutaneous disorders including skin cancers. Experimental studies have demonstrated that UV radiation can act both as a tumor initiator and tumor promoter (24,25). UV radiation is a potent inducer of ROS, and these ROS are responsible for the photooxidative damage to nucleic acids, lipids, proteins and also result in phosphorylation and activation of MAPK and NF-κB pathways (1,26–29). In recent years, considerable attention is focused on the use of naturally occurring botanicals for their potential preventive effect against UV radiation–mediated damages that we refer to as "photochemopreventive effects" (1). Botanical agents, which generally contain agents that are antioxidant in nature, are being widely used in skin care products and even in customized diets and beverages. Among these botanicals, PFE derived from the tree *P. granatum* is a rich source of anthocyanins and hydrolyzable tannins and possesses potent antioxidant and anti-inflammatory properties. The molecular mechanism(s) by which PFE imparts its photochemopreventive effects against UV-mediated damages are not completely understood. In this study, we determined the photochemopreventive potential of PFE on UV-B–mediated phosphorylation and activation of MAPK and NF-κB pathways in NHEK. Studies have demonstrated that inhibition of the phosphorylation of MAPK may prevent the downstream events such as activation of NF-κB pathway that would lead to the prevention of photocarcinogenic events in the skin (2).

MAPK encompass a large number of serine/threonine kinases involved in regulating a wide array of cellular processes including proliferation, differentiation, stress adaptation and apoptosis (30,31). The MAPK are divided into three multimember subfamilies: ERK1/2, JNK1/2 and p38. Studies have shown that ERK, JNK and p38 subfamilies are activated in response to oxidant injury and therefore could potentially contribute to influence cell survival. Studies have demonstrated that exposure of human keratinocytes to physiologic doses of UV-B activates EGFR/ERK1/2 and p38 signaling pathways via ROS generation (32,33). UV-B–induced phosphorylation of MAPK pathway has been shown to be inhibited by the use of antioxidants, thereby suggesting that MAPK are important targets affected by ROS (8). Studies have revealed that sublethal doses of UV-B are potent inducers of the JNK/SAPK family of MAPK but only weak activators of ERK (34). Low-dose UV-B irradiation of normal human skin induces rapid and reversible phosphorylation of JNK and p38. Because PFE possesses potent antioxidant and antiproliferative properties, we first determined the photochemopreventive effect of PFE on UV-B–mediated phosphorylation of MAPK in NHEK. In this study, we found that phosphorylation of ERK1/2 after UV-B exposure was observed for a shorter time period in comparison with JNK1/2 and p38. Our data demonstrated that pretreatment of NHEK with PFE inhibited UV-B–mediated phosphorylation of ERK1/2, JNK1/2 and p38 (Fig. 1A,B). In addition, a distinct level and time point of phosphorylation and inhibition of MAPK by PFE treatment suggests independent mechanisms of regulation of each pathway in NHEK. Studies have demonstrated that both p38 and ERK were required for UV-B–induced c-*fos* expression in human keratinocytes HaCaT. Inhibition of both p38 and ERK simultaneously completely abrogated UV-B–induced c-*fos* transcription and downregulated basal transcription of the c-*fos* gene (35,36). Studies have shown that JNK/SAPK, ERK1/2 and p38 pathways respond to proinflammatory cytokines and oxidative and environmental stress resulting in transcriptional activation of NF-κB (2,37,38).

NF-κB is an ideal target for development of anti-inflammatory agents because it regulates the transcription of various genes that are involved in inflammation, blocks apoptosis and promotes cell proliferation, tumor promotion, angiogenesis and metastasis (39,40). NF-κB is present in the cytosol as a heterodimer usually consisting of its p50 and p65 subunits bound to its inhibitory proteins IκB (41). NF-κB can be activated by a wide range of stimuli that includes UV light, inflammatory cytokines, phorbol esters, lipopolysaccharides and a variety of mitogens (2,11,41). Studies have shown that these stimuli activate IκB kinases (IKK) either directly or indirectly and are central to NF-κB activation (42,43). The activated IKK phosphorylates the inhibitory κB proteins on either serine residue 32 or 36 of IκBα (44,45). The released NF-κB then moves from the cytoplasm to the nucleus and activates transcription of target genes (46). Therefore, development of novel NF-κB inhibitory drugs is important for prevention and possible treatment of skin cancer. Botanical antioxidants of food substances have a great advantage over existing drugs because they are nontoxic and inexpensive. In this investigation, the effect of PFE on the pattern of NF-κB/p65 activation and its nuclear translocation induced by UV-B radiation in NHEK was defined to assess its mechanism of action. Studies have demonstrated that antioxidant activity of PFE, although vary depending upon the variety, is higher than that of red wine and green tea (15). PFE has shown potent antioxidant capacity against lipid peroxidation that may be a central link for the antiatherogenic effect of PFE on lipoprotein, macrophages and platelets (47,48). Compounds that scavenge ROS inhibit NF-κB activation (29). In this study, we analyzed the UV-B–induced phosphorylation on endogenous NF-κB/p65 at Ser^{536}. We demonstrated that NHEK irradiated with UV-B resulted in phosphorylation of p65 at Ser^{536} in the cytoplasm. As evident from Western blot analysis, we found that pretreatment of NHEK with PFE resulted in a dose- and time-dependent inhibition of UV-B–mediated phosphorylation of NF-κB/p65 at Ser^{536} (Fig. 2A,B).

We next assessed the effect of PFE on UV-B–mediated activation and nuclear translocation of NF-κB in NHEK using Western blot analysis, ELISA and NF-κB/p65 DNA-binding EMSA assays. Interestingly, we found that pretreatment of NHEK with PFE before UV-B radiation significantly inhibited UV-B–mediated activation and nuclear translocation of NF-κB (Figs. 2A,B and 3A,B) and NF-κB DNA-binding activity (Fig. 4A,B) in a dose- and time-dependent manner. Our results showed a positive correlation between NF-κB/p65 activation and its translocation to the nucleus (Figs. 2 and 3) and phosphorylation and degradation of IκBα in the cytoplasm in NHEK after UV-B exposure (Fig. 5A,B). Interestingly, we found that pretreatment of NHEK with PFE before UV-B irradiation

significantly inhibited UV-B–induced NF-κB activation (Figs. 2–4) and phosphorylation and degradation of IκBα protein (Fig. 5A,B) in a dose- and time-dependent manner. Because PFE inhibited IκBα phosphorylation and degradation, this study suggests that the effect of PFE on NF-κB/p65 is through inhibition of phosphorylation and subsequent proteolysis of IκBα. Our study suggests that modulation of IKKα by PFE is important because IKK complex is believed to be an important site for integrating signals that regulate the NF-κB pathway (Fig. 5A,B). NF-κB controls the expression of several growth factors, oncogenes and tumor suppressor genes (c-myc, p53), genes encoding cell adhesion proteins (ICAM-1, ELAM-1, VCAM-1) and proteases of the extracellular matrix (49). Studies have shown that NF-κB activity affects cell survival and determines the sensitivity of cancer cells to cytotoxic agents as well as ionizing radiation (50).

In summary, our results suggest that treatment of NHEK with PFE before UV-B exposure resulted in a dose- and time-dependent inhibition of UV-B–mediated activation and phosphorylation of MAPK and NF-κB pathways. On the basis of our data, we suggest that PFE may have the potential to protect against the adverse effects of UV-B radiation. These results provide molecular basis to conduct in-depth studies and to establish active components present in PFE responsible for photochemopreventive effects.

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