UV radiation down-regulates Dsg-2 via Rac/NADPH oxidase-mediated generation of ROS in human lens epithelial cells

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Abstract. Ultraviolet radiation-induced cataract has been believed to be associated with degradation of cellular components. We report that, in cultured human lens epithelial cells, UV radiation analogous to H_2O_2 treatment down-regulates desmosomal protein desmoglein-2. UV radiation induces generation of reactive oxygen species and transiently activates epidermal growth factor receptor, which in turn induces translocation of Rac2 and NADPH oxidase activity. Collectively, our data demonstrate that UV-induced desmoglein-2 down-regulation is mediated reactive oxygen species which are generated through EGFR activation and Rac2/NADPH oxidase activation, suggesting that antioxidants may be applied for protection against UV-induced cataract.

Introduction

Among many environmental health risk factors, ultraviolet radiation (UV) is one which has increased for the past few decades due to the depletion of ozone layers. Accordingly, unprecedented attention has been made, despite human behavior remaining unchanged, to the fact that solar UV radiation causes human skin aging and skin cancer (1,2). However, as of yet, underpaid attention has been given to the fact that UV also induces eye damage such as cataracts. While skin care products are abundant, eye care products are hard to find. Accumulating epidemiologic and animal studies suggest that UV radiation may be cataractogenic (3,4), although the cellular processes involved in such cataractogenesis are yet to be elucidated (5). Understanding the molecular mechanisms through which UV radiation induces cataracts may help provide better clinical management and ultimately prevent such an occurrence.

An immediate consequence of UV radiation is the production of reactive oxygen species (ROS), including hydrogen peroxide, which are proven to be deleterious to DNA, lipids and proteins in the lens, probably leading to cataract (6). Hydrogen peroxide at concentrations found in some cataract patients reportedly induces both lens epithelial cell apoptosis and cortical opacity. Also, temporal and spatial distribution of induced apoptotic lens epithelial cells precedes development of lens opacification (7,8). While lens epithelial cell apoptosis is considered as a common cellular basis for initiation of cataract formation (7,8), increasing data supports the notion that imbalanced cellular redox status is a potent pathogenic factor that leads to cellular dysfunctions resulting in various degenerative diseases, such as cataract.

Desmosomes, composed of a number of proteins including desmogleins, are present in lens cells and also a component of the subplasmalemmal coat of these cells, and highly organized intercellular junctions that provide mechanical integrity to tissues by anchoring intermediate filaments to sites of strong adhesion (9-12). Defect in the desmosomal and gap junction structure most prominently affecting epithelia is associated with defect in cellular functions (13). Furthermore, pathological studies have demonstrated that desmoglein expression may play an important role in the invasiveness and metastatic activity of human endometrial carcinoma, which may therefore be used as differentiation markers for endometrial carcinoma (14).

In our preliminary study, we observed that UV radiation induces generation of ROS and both UV and H_2O_2 down-regulate Dsg-2 in cultured human lens cells. However, neither the mechanisms through which UV induces generation of ROS nor the mechanism through which UV down-regulates the Dsg-2 of lens cells have been addressed. We propose that UV-induced Dsg2 down-regulation is mediated by ROS. We

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Abbreviations: HLECs, human lens epithelial cells; UV, ultraviolet; EGFR, epidermal growth factor receptor; NADPH oxidase, nicotine adenine dinucleotide phosphate (H) oxidase; ROS, reactive oxygen species; Dsg-2, desmoglein-2; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate

Key words: NADPH oxidase, reactive oxygen species, desmogleins, desmosome, ultraviolet

present evidence that UV activates EGFR, induces Rac2 translocation from the cytoplasm to the membrane and activates NADPH oxidase which generates ROS leading to Dsg-2 down-regulation.

Materials and methods

UV light apparatus. As with previous studies (1,15), the UV-irradiation apparatus used in this study consisted of four F36T12 UV tubes. A Kodacel TA401/407 filter was mounted 4 cm in front of the tubes to remove wavelengths <290 nm (UVC). Irradiation intensity was monitored using an IL443 phototherapy radiometer and a SED240/UV/W photodetector. Before UV irradiation, cells were washed with 1 ml PBS and changed to 0.5 ml PBS in each well. Cells were irradiated at a desired intensity without a plastic dish lid. After UV irradiation, cells were returned to incubation in basal DMEM with treatments for various time points prior to harvesting.

Cell culture. Human lens epithelial cells (cell line) were obtained from Dr Fu Shang of Tufts University. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 20% fetal bovine serum (Hyclone), penicillin/streptomycin (1:100, Sigma) and 4mM L-glutamine, in a humid atmosphere incubator with 5% CO_2 at 37˚C. Cells were reseeded in 6-well plates at a density of $0.2x10^6$ cells/ml with fresh complete culture medium. Unless otherwise indicated, cultures were grown to 70-80% confluence and then serum-starved overnight in DMEM medium prior to treatment.

Antibodies. Anti-phospho-EGFR (Tyr1068) antibody was from Cell Signaling Technology (Beverly, MA). Anti-ß-actin was purchased from Sigma. Anti-Rac2, anti-desmoglein-2, anti-EGFR (1005), goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP antibody and goat anti-rabbit-IgG-FITC were from Santa Cruz Biotechnology (Santa Cruz, CA).

Treatment of human lens epithelial cells. Cells were treated with indicated dosages of UV or concentrations of H_2O_2 and remained in the medium for the remainder of the experiment. The EGF solution was diluted in DMEM medium without serum to a final concentration of 100 ng/ml. In the time-dependent experiments, HLECs were treated with 100 ng/ml of EGF and harvested at 5 min, 15 min, 30 min, 1 h, and 2 h after EGF treatment.

Western blot analysis. As reported previously (16), cultured human lens epithelial cells with and without treatment were washed with cold PBS containing 50 mM phosphate, pH 7.4, 100 mM NaCl, and 10 mM KCl), and harvested by scraping into 150 µl RIPA buffer containing 50mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1mM EDTA, 0.25% sodium deoxycholate, 1 mM NaF, 10 µM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 µM pepstatin). Cell lysates were incubated in a cold room (4˚C) for 30 min. After centrifugation at 14,000 rpm for 10 min at 4˚C, the protein concentration was determined by a Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins (30 µg) were denatured in 2X SDS-PAGE sample buffer for 5 min at 95˚C. The proteins were separated by 12%, 10% or 7.5% SDS-PAGE and electro-transferred to Immobilon-P membrane (Millipore, Bedford, MA) for 2 h at 4˚C. Nonspecific binding was blocked with 10% dry milk in TBST (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.01% Tween-20) for 1 h at room temperature. After blocking, the membranes were incubated with specific antibodies against EGFR, phospho-EGFR, at appropriate dilutions in dilution buffer (2% BSA in TBST) overnight at 4˚C. The blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000, Santa Cruz Biotechnology) at room temperature for 1 h. Antibody binding was detected using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) following the manufacturer's instructions and visualized by autoradiography with Hyperfilm.

FACS analysis. Cultured human lens epithelial cells were deprived of serum for 24 h and loaded with 1 µM of a fluorescent dye, dihydrorhodamine (DHR) that reacts with ROS in cells and results in fluorescence. Cells were irradiated with UV (30 mJ/cm^2), and were then collected and fixed, the generation of ROS of treated cells was measured by FACS analysis as described previously (1). Induction of ROS generation was expressed in arbitrary units.

Immunofluorescence of Rac2 translocation. Cells were seeded in 4-well chamber slides (Fisher Scientific, USA) at a density of $1x10^5$ cells/ml with fresh complete culture medium (0.25 ml per well), and incubated for 24 h in a CO^2 incubator. Cells were grown to 70-80% confluence and then serum-starved overnight in DMEM medium prior to UV (30 mJ/cm^2) or EGF treatment (100 ng/ml), and collected at 5, 15, 30 and 60 min. After treatment, the cells were washed with cold Dulbecco's phosphate-buffered saline (PBS). The cells were then fixed with cold methanol for 10 min and washed with cold PBS, incubated with 250 µl of 1% BSA in PBS for 1 h at room temperature, washed twice with PBS for 15 min, and incubated with anti-Rac2 (dilution, 1:100) in 1% of BSA in PBS for 2 h at room temperature. After washing with PBS, cells were incubated with FITC-conjugated secondary antibody (dilution, 1:100) in 1% BSA in PBS for 1 h at room temperature. Cells on the glass slide were observed by fluorescence microscopy (17).

NADPH oxidase assay. As described previously (1,17), UV-irradiated or EGF-treated cells were washed twice in cold Dulbecco's phosphate-buffered saline and were scraped from the plate in the same solution followed by centrifugation at 750 x g, 4˚C, for 10 min. The pellet was re-suspended in buffer containing 20 mM KH_2PO_4, 1 mM EGTA, 10 µg/ml aprotinin, 25 µg/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride. The cell suspension was homogenized with 50 strokes in a Dounce homogenizer on ice, and aliquots of the homogenate were used immediately. To start the assay, 100 µl of homogenate was added to 900 µl of 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 500 µM luciginin, and 100 µM NADPH. NADPH oxidase activity was expressed in arbitrary units.

Statistical analysis. The values in the figures are expressed as the means ± standard deviation (SD). In the experiments

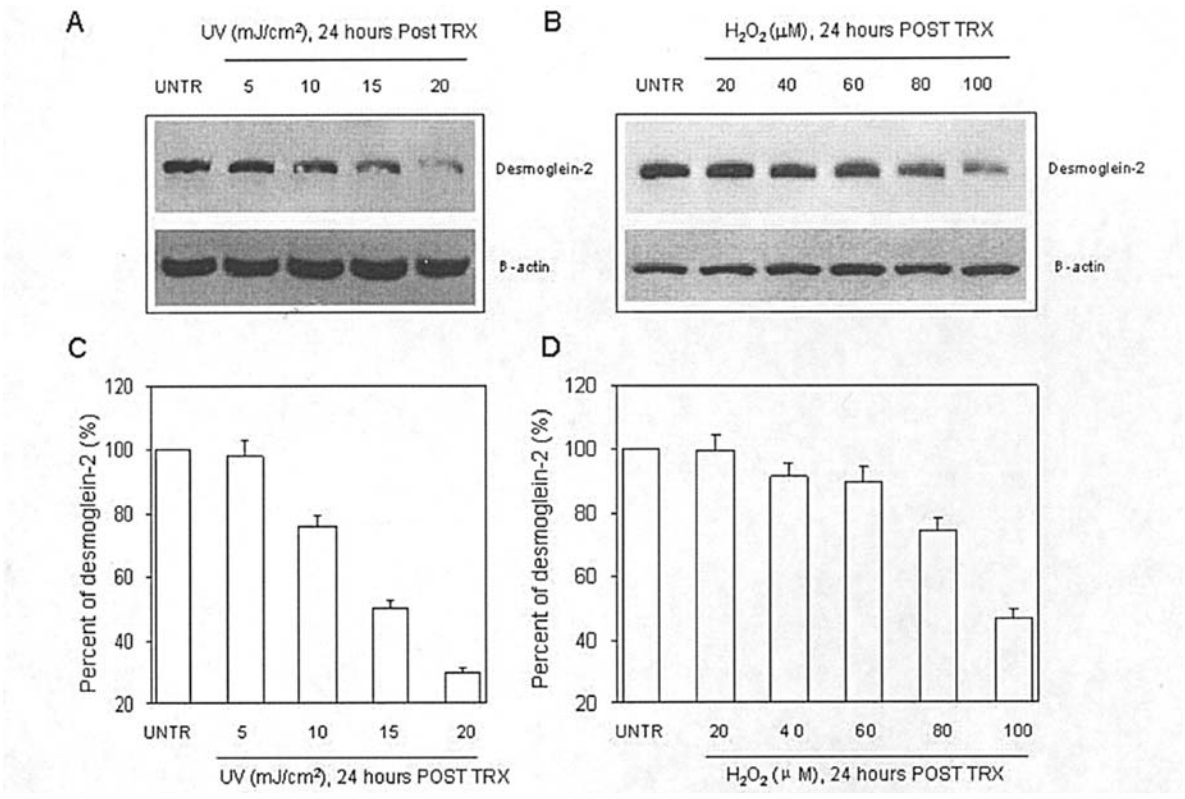

Figure 1. UV and H_2O_2 induce desmoglein-2 down-regulation in cultured human lens epithelial cells. Cells were treated with UV (5, 10, 15, 20 mJ/cm^2) or H_2O_2 (20, 40, 60, 80, 100 μM) and harvested at 24 h after treatment. Desmoglein-2 expression in cell lysates was analyzed by Western blotting (A and B). The percentage of Dsg-2 of UV- and H_2O_2-treated cells compared to untreated controls (UNTR) is shown in C and D, where the data are the mean ± SD of triplicate experiments.

involving UV and H_2O_2-induced desmoglein-2 down-regulation, ROS generation, EGFR phosphorylation, and NADPH oxidase assay, are representative of three different experiments are shown. Statistical analysis of the data between the control and treated groups was performed by Student's t test. Values of $P<0.05$ were considered as significant.

Results

UV radiation induces desmoglein-2 down-regulation in cultured human lens epithelial cells. Desmosomes, which are composed of a number of proteins, are important for cell-cell interaction. To test whether UV down-regulates desmosomal proteins, we first treated cells with UV (15 mJ/cm^2) irradiation and then detected the expression of desmosomal proteins by Western blot analysis. The data using existing antibodies show that, in cultured human lens cells, desmoglein-2 (Dsg-2) but not desmoglein-1 (data not shown) is expressed. As shown in Fig. 1A and B, UV and H_2O_2 induce Dsg-2 down-regulation in a dose-dependent manner. Dsg-2 expression decreases to 29.7% and 46.8% of untreated cells, respectively, 24 h post treatment with UV (20 mJ/cm^2) and H_2O_2 (100 μM) treatment (Fig. 1C and D). The results clearly demonstrate that UV and H_2O_2 down-regulate Dsg-2 expression in cultured human lens epithelial cells.

UV induces generation of reactive oxygen species in cultured HLECs. The above data indicates that UV and H_2O_2 induce desmoglein-2 down-regulation in human lens cells. To study whether UV radiation induces generation of ROS, we directly detected the generation of ROS following UV irradiation in cultured human lens cells using FACS analysis. Human lens cells were loaded with DHR (1 μM) for 2 h, and treated with UV (30 mJ/cm^2) or H_2O_2 as positive control (500 μM), collected and fixed at 5, 15, 30, 60, and 120 min post treatment for FACS analysis. The data shows that UV (30 mJ/cm^2) induces ROS generation in a time-dependent manner, ROS appears at 5 min after treatment, peaks at 15 min after treatment, and then remains elevated for 2 h (Fig. 2A and B). The data indicates that UV induces ROS generation in cultured human lens cells with a pattern similar to that observed in cultured human keratinocytes, as previously reported (1).

UV induces NADPH oxidase activity in cultured HLECs. The above data shows that UV induces generation of ROS in cultured human lens cells. ROS are generated through various pathways, including Rac/NADPH oxidase-mediated pathway. To test the hypothesis that UV-induced ROS is mediated by NADPH oxidase in culture human lens cells, we directly measured NADPH oxidase activity post UV radiation. Cells were treated with UV (30 mJ/cm^2) and harvested at different time points (5, 15, 30, 60 min), and NADPH oxidase in cell lysates was analyzed. The data shows that NADPH oxidase activity starts to increase 5 min post UV treatment, the activity peaks at 30 min, and remains elevated for approximately 1 h (Fig. 3A). As expected, EGF treatment (100 ng/ml) also induces NADPH oxidase activity in a time-dependent manner (Fig. 3B). These results demonstrate that UV radiation induces

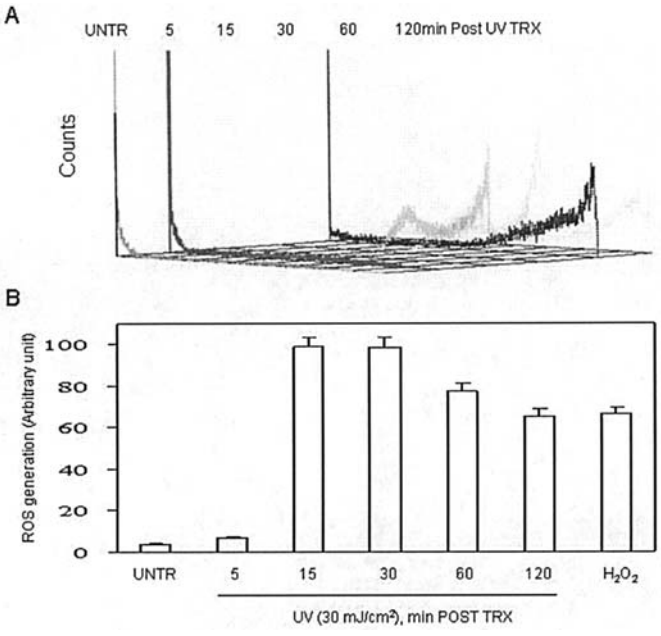

Figure 2. UV induces generation of reactive oxygen species in cultured HLECs. Human lens epithelial cells were loaded with DHR (1 μM) for 2 h, and treated with UV (30 mJ/cm^2) or H_2O_2 (500 μM) as positive control, collected and fixed at 5, 15, 30, 60, and 120 min post treatment for FACS analysis (A). Induction of ROS generation was expressed in arbitrary units (B) as the mean ± SD of triplicate experiments.

NADPH oxidase activity in cultured human lens epithelial cells.

UV induces Rac2 translocation in cultured HLECs. The data above show that UV induces ROS generation and activation of NADPH oxidase in cultured human lens epithelial cells. Upon EGF treatment, one of the subunits of NADPH oxidase, Rac2, translocates from the cytoplasm to the cell membrane, resulting in NADPH oxidase activation. To investigate whether UV radiation induces Rac2 translocation, we labeled cells following UV radiation with Rac2-specific antibody and used immunofluorescence microscopy for observation. Cells were cultured in chamber slides and radiated with UV (30 mJ/cm^2), and fixed at 5, 15 and 30 min after UV treatment, and photographed using a microscope after immunofluorescence staining. The data shows that, before UV radiation, Rac2 proteins are largely distributed in the cytoplasm, and translocate to the cell membrane 5 min post UV treatment and return to the cytoplasm within 30 min (Fig. 4 upper panel). As expected, EGF treatment also induces Rac2 translocation in cultured human lens cells (Fig. 4 lower panel). These results indicate that UV radiation induces Rac2 protein translocation from the cytoplasm to the cell membrane, resulting in NADPH oxidase activation in cultured human lens epithelial cells.

UV induces EGF receptor phosphorylation in cultured HLECs. EGF binds to EGFR leading to autophosphorylation of EGFR, followed by Rac2 translocation and NADPH oxidase activity. The above data indicates that UV radiation induces NADPH oxidase activation and Rac2 translocation. To test whether EGFR activation is mediated by UV-induced Rac2 translocation, we directly measured EGFR phosphorylation after UV radiation in human lens cells. Cells were deprived of serum overnight and exposed to UV (30 mJ/cm^2) and harvested at 5, 15, 30, 60 and 120 min after treatment. Western blot analysis using antibody recognizing phospho-EGFR indicates that UV radiation induces EGFR tyrosine phosphorylation in a time-dependent manner. EGFR phosphorylation starts 5 min post UV radiation, the activity peaks at 15 min and remains elevated for 30 min (Fig. 5A and C). As expected, EGF treatment induces EGFR phosphorylation in a time-dependent manner. EGFR phosphorylation peaks at 5 min and remains elevated for 30 min (Fig. 5B and D). The data demonstrates that UV induces EGFR phosphorylation in cultured human lens cells.

Discussion

Human lens defense mechanisms against UV radiation result in protein expression via the induction of genes and their transcriptional regulation, where the molecular bases for abnormalities are associated with cataracts leading to the development of lens opacities (6,18-22). Cataracts have been also found to be associated with proteolytic degradation (23). For example, UV induces protein and lipid peroxide damage

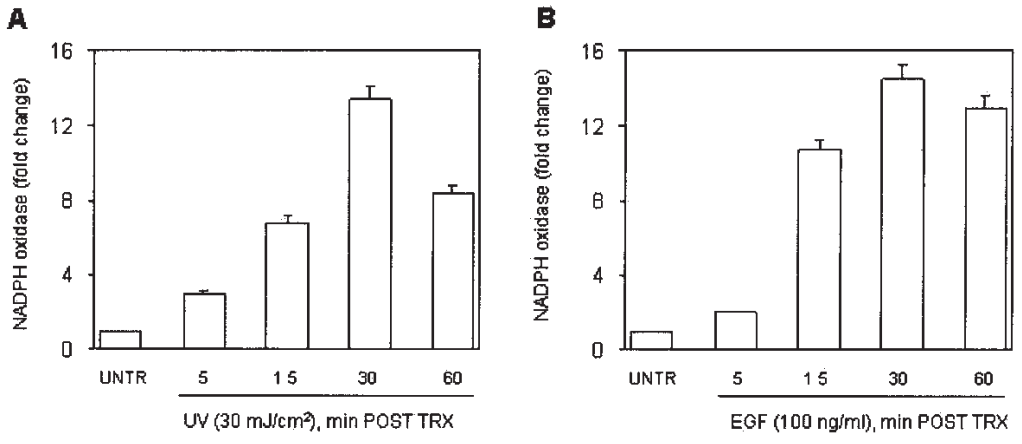

Figure 3. UV and EGF induce NADPH oxidase activity in cultured HLECs. Cells were treated with UV (30 mJ/cm^2) or EGF (100 ng/ml), and harvested at different time points (5, 15, 30, 60 min). NADPH oxidase activity in cell lysates was analyzed as described in Materials and methods. The activity was expressed in arbitrary units (A and B) with the mean ± SD of triplicate experiments.

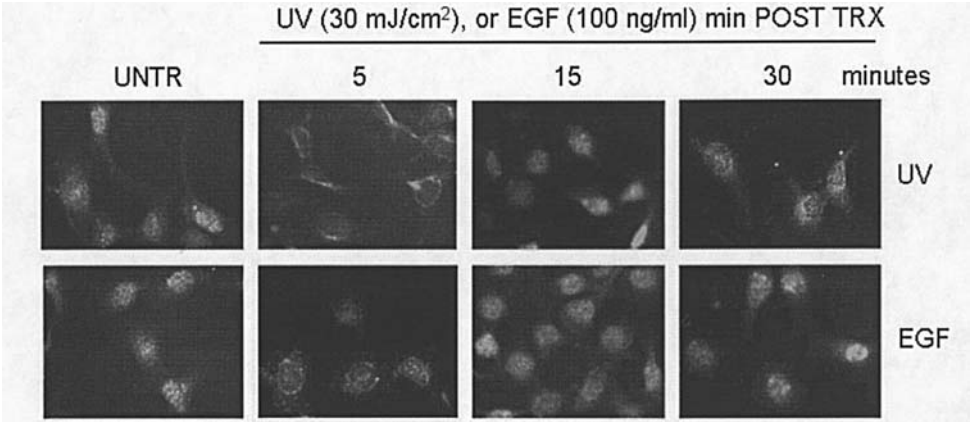

Figure 4. UV and EGF induce Rac2 translocation in cultured HLECs. Cells were irradiated with UV (30 mJ/cm^2) or EGF (100 ng/ml) and fixed at 5, 15, 30 and 60 min after treatment. The cells were labeled with anti-Rac2 antibody and FITC-conjugated secondary antibody. Cells on the glass slide were observed under a fluorescence microscope.

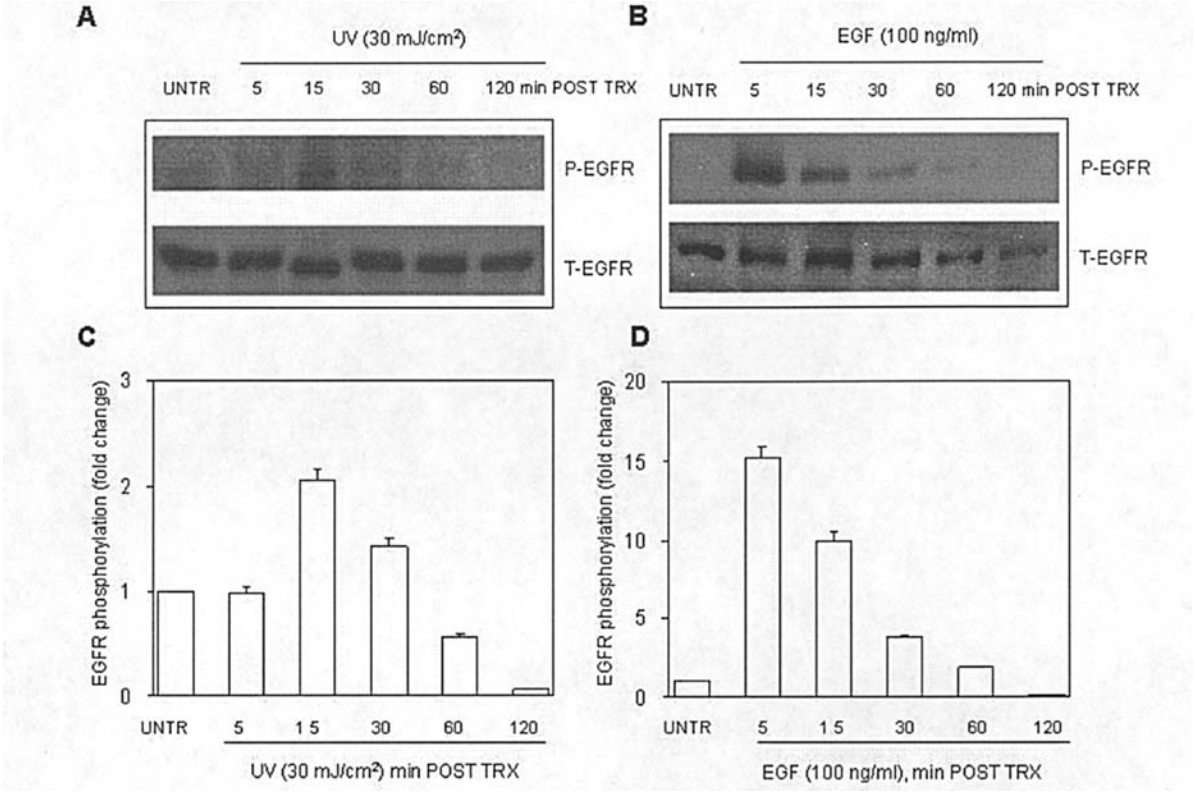

Figure 5. UV and EGF induce EGF receptor phosphorylation in cultured HLECs. Cells were treated with UV (30 mJ/cm^2) and EGF (100 ng/ml), and harvested at different time points (5, 15, 30, 60, and 120 min), phospho-EGFR and total EGFR in cell lysates were analyzed by Western blotting (A and B). EGFR phosphorylation was expressed in arbitrary units (C and D) as the mean ± SD of triplicate experiments.

in lens culture, increases production of ß-amyloid precursor protein and ß-amyloid in mammalian lenses, and contributes to the mechanism by which oxidative damage leads to lens opacification. Although UV-induced rapid apoptosis is considered to be associated with the initiation of cataracts (24,25), accumulating data suggest that ROS are involved in protein degradation, and UV-induced cataract formation is closely related to the accumulation of oxidatively altered proteins (19,20,23). Our data demonstrates that, similar to H_2O_2, UV radiation down-regulates Dsg-2 (Fig. 1) and Collagen Type I (data not shown) in cultured human lens cells, suggesting that ROS are involved in UV-induced Dsg-2 down-regulation.

UV radiation induces ROS generation in cultured human keratinocytes and fibroblasts *in vitro* and in human skin *in vivo* (1,26). In this study, we demonstrate that UV also induces generation of ROS in cultured human lens cells (Fig. 2). The mechanism through which ROS are generated is not understood. We found that NADPH oxidase is involved in UV-induced ROS production in human lens cells (Fig. 3), which is consistent with our previous finding that UV activates NADPH oxidase in both cultured human keratinocytes *in vitro* and in human skin *in vivo* (1,27). These data suggest that NADPH oxidase may be universally involved in generation of ROS in response to a variety of stimulants.

NADPH oxidase, composed of several subunits, was first found to be involved in the bursting of ROS in immune cells in response to infections (28). Subunits of NADPH oxidase have also been expressed in various cell types, including human skin keratinocytes and fibroblasts (1) and cultured human lens cells (Fig. 4). Upon growth factor receptor activation, one of the subunits of NADPH oxidase, namely Rac2, translocates from the cytoplasm to the cell membrane, docking with other members of the enzyme complex, leading to the activation of the enzyme. In this study, we found that UV radiation also induces Rac2 transient translocation (Fig. 4) in a similar manner to EGF treatment, which is consistent with our previous finding in cultured human keratinocytes (1).

Compelling evidence supports the notion that EGFR is involved in UV signaling leading to the activation of kinase cascade and a number of transcription factors (1,2,26,29). The mechanism of UV-induced EGFR activation remains to be elucidated (30). We recently provided evidence that EGFR activation in response to UV radiation is mediated by shedding of ectodomain of EGFR ligands possibly through matrix and/or membrane proteolytic enzymes (15). EGFR activation plays an important role in NADPH oxidase activity (1). In this study, we observed that UV radiation also activates EGFR in the cultured human lens (Fig. 5), further supporting the above notion that EGFR remains one of the major undisputable signaling transmission molecules from the extra-cellular compartment to the cytoplasm, leading to the activation of downstream components, such as kinases and transcription factors.

Desmosomes are highly organized intercellular junctions that provide mechanical integrity to tissues by anchoring intermediate filaments to sites of strong adhesion. Desmosome assembly and disassembly are regulated post-translationally by calcium, kinase/phosphatase activity, proteolytic processing, and cross-talk with adherens junctions. These proteins participate in various nuclear functions; in some cases, transducing signals regulating cell growth and differentiation (31). In epithelial cells, desmosomes represent a second type of anchoring junctions mediating strong cell-cell contacts (9,10,32). Down-regulation of desmogleins may play an important role in the invasiveness and metastatic activity of cancer cells (14). The consequence of UV-induced Dsg-2 down-regulation, or the linkage between degradation of Dsg-2 and cataract, remains to be further investigated.

In conclusion, we demonstrate in this study that UV radiation down-regulates desmoglein-2 in cultured human lens cells that may be involved in UV-induced cataracts. We provide evidence that UV radiation activates EGFR, which in turn induces Rac2 translocation and NADPH oxidase activity leading to ROS generation and degradation of Dsg-2. These data suggest that EGFR inhibitor and antioxidants may be used for protection against UV-induced Dsg-2 down-regulation and cataract formation.

Acknowledgments

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