Ultraviolet Irradiation-Induced Volume Alteration of Corneal Epithelial Cells

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PURPOSE. The purpose of the study is to understand how extracellular stresses, such as ultraviolet (UV) irradiation, affect corneal epithelial cells. Cell volume changes, damage to corneal epithelial integrity, and cellular responses were assessed after exposure to UVC stresses.

METHODS. Primary human and rabbit corneal epithelial cells were exposed to UVC light in culture conditions. Ultraviolet C irradiation–induced changes in cell size and volume were measured by real-time microscopy and self-quenching of the fluorescent dye calcein, respectively. The effects of UVC irradiation on *Src* and focal adhesion kinase (FAK) phosphorylation and FAK-dependent integrin signaling were detected by ELISA, immunoblotting, and immunostaining.

RESULTS. Ultraviolet C irradiation induced both size and volume shifts in human and rabbit corneal epithelial cells. Ultraviolet C irradiation-induced decrease of cell volume elicited activation of *Src* and FAK, characterized by increased phosphorylations of Src^{Y416}, FAK^{Y397}, and FAK^{Y925}. In addition, immunostaining studies showed UVC irradiation–induced increases in phosphorylation of FAK and formation of integrin β5 clustering. Application of Kv channel blockers, including 4-aminopyridine (4-AP), α-DTX, and depressing substance-1 (BDS-1), effectively suppressed UVC irradiation–induced cell volume changes, and subsequently inhibited UVC irradiation–induced phosphorylation of *Src*/FAK, and formation of integrin β5 clustering, suggesting UVC irradiation–induced volume changes and *Src*/FAK activation. Hyperosmotic pressure–induced volume decreases were measured in comparison with effects of UVC irradiation on volume and *Src*/FAK activation. However, Kv channel blocker, 4-AP, had no effect on hyperosmotic pressure–induced responses.

CONCLUSIONS. The present study demonstrates that UVC irradiation–induced decreases in cell volume lead to *Src*/FAK activation due to a rapid loss of K ions through membrane Kv channels.

Keywords: FAK, *Src*, volume changes, K^+ channel blockers, cytoskeleton remodeling

The maintenance of corneal epithelial barrier function requires a continuous renewal process to slough off terminally differentiated and to provide a protective mechanism against noxious agents and infections.[1] The physiological balance in the renewal process between the ability of the corneal epithelium to proliferate and differentiation contributes to the maintenance of corneal deturgescence, transparency, and normal vision function.[2–5] There are reports indicate ultraviolet (UV) B (150–225 mJ/cm²) or UVC (25–40 μJ/cm²) can effectively activates K^+ channel and K^+ fluxes in the membrane of corneal epithelial cells.[6–9] Ultraviolet irradiation–induced rapid loss of intracellular K^+. Exposure of corneal epithelial cells to results in a series of cellular responses including increases in vitamin D3 synthesis and activations of multiple signaling pathways and apoptosis.[10–14] Ultra violet irradiation–induced increases in cell membrane Kv channel activity results in activations of the signaling pathways, including the Erk, JNK, and p38 pathways.[7,11] In addition, activation of nuclear factor kappa-B (NFκB) by TNF-α promotes cell survival in UV irradiation–induced corneal epithelial cells, suggesting a variation of this pathway in corneal epithelial cells.[10] Suppression of Kv channel activity with specific Kv channel blockers significantly inhibits UV irradiation–induced activation of mitogen-activated protein (MAP) kinase cascades, but does not prevent hyperosmotic stress–induced MAP kinase activation.[11] There are more evidence indicating that cell volume changes by hyperosmotic shock stimulate activation of JNK and p38 signaling pathways.[7,11,15–18] Interestingly, activation of MAP kinases by volume changes is not fully dependent on hyperosmotic conditions instead that it is a volume-dependent process.[19]

The integrity and function of corneal epithelial cells is regulated by activation of various growth factor receptors, membrane ion channel/transport, and intracellular pathways. Previous studies demonstrate that UV irradiation can activate cytokine receptor and Kv channel activity to evoke specific signaling pathways that affect the physiological function including regulation of corneal epithelial fate and corneal epithelial wound healing.[20,21] In addition, X-ray irradiation can also activate K^+ channels in A549 and HEK293 cells.[22] Inhibition of K^+ efflux by using Kv channel blockers or by increasing extracellular K^+ concentrations inhibit stress-in-

duced activation of signaling pathways and apoptosis in many cell types including corneal epithelial cells.[23-26] The Kv channel type activated by UV irradiation and hypoxic stress in corneal epithelial and other cells has been determined to be the Kv3.4 and Kv2.1 Kv channels by using electrophysiological and biochemical approaches.[27,28] These combined approaches used in corneal epithelial cells include screening K^+ channels with a panel of specific anti-K^+ channel antibodies, applying specific channel blockers, and analyzing whole-cell and single-channel characteristics.[27]

It has been shown that cell shrinkage at the leading edge of cells resulting in a partial detachment activates the integrin signaling because integrins first engage their ligands in the leading edge region, resulting in activation of scaffold proteins focal adhesion kinase (FAK) and paxillin, as well as *Src*.[29-31] In the present study, we focus on the study of UVC irradiation–elicited early responses and signaling events involved in corneal epithelial cells. The hypothesis is based on that UVC irradiation induced a significant increase in K^+ efflux as one of the early events that can lead cell shrinkage resulting in activation of FAK-dependent integrin pathway.[32] We use Kv channel blockers to inhibit UVC irradiation–activated Kv channels to prevent UVC irradiation–induced cell volume change, and to suppress UVC irradiation–induced activation of the FAK-dependent integrin pathway.

MATERIALS AND METHODS

Culture and UVC Exposure of Corneal Epithelial Cells

Human and rabbit corneal epithelial cell lines used in various studies have been well characterized in previous studies.[33-35] In the current study, two kinds of the corneal epithelial cells were used in the study including the primary human corneal epithelial cells and rabbit corneal epithelial cells. Primary human and rabbit corneal epithelial cells were cultured in a serum-free keratinocyte medium (Defined Keratinocyte-SFM; Invitrogen, Carlsbad, CA, USA), and primary human corneal epithelial cells were grown in Dulbecco's modified Eagle's medium/F-12 (1:1; Gibco, Waltham, MA, USA) containing 10% fetal bovine serum and 5-μg/mL insulin. Cells were cultured in an incubator supplied with 95% air and 5% CO_2 at 37°C. The medium was replaced every 2 days, and cells were subcultured by treatment of cells with 0.05% trypsin-EDTA (Gibco). Primary human corneal epithelial cells were obtained by passing fresh isolated limbal stem/progenitor cells into collagen/fibronectin (50%/50%)-coated surface and an airlift (reduced medium level) procedure to induce differentiation. The morphologic changes were closely monitored. The medium contains 10% fetal bovine serum, 5-mg/mL insulin, and 10,000 units/mL penicillin, and 10,000 μg/mL streptomycin, and maintained in an incubator supplied with 95% air and 5% CO_2 at 37°C. The medium was replaced every 2 days and cells were passed by treating cells with 0.05% Trypsin-EDTA. For UVC irradiation experiments, corneal epithelial cells were exposed to UVC light at an intensity of 45 μJ/cm².[8] In previous studies, we found that stimulation of corneal epithelial cells with UVC at 45 μJ/cm² has a very similar or compatible effect as UVB at a dosage of 200 mJ/cm².[6] The reason that we used UVC is that a quick stimulation allowed us to record the fast response of cells under a real time microscope (see below). The UVC source was from an 84-Watt UVC lamp with a wavelength of 254 nm (Spectronic Corp, Westbury, NY, USA). The intensities of the UVC light were calibrated by using an optical radiometer (MS-100; UVP LLC, Upland, CA, USA). Hyperosmotic stress stimulation was performed by adding various concentrations of sorbitol to the culture media following

a time course. Osmotic pressures of hyperosmotic media were measured by using a Vapor Pressure Osmometer (VAPRO5520). Human tissue was handled in accordance with the tenets of the Declaration of Helsinki. The experimental protocol was exempted by the University of California Los Angeles institutional review board (Los Angeles, CA, USA).

Live Cell Imaging and Analysis

Morphologic changes of corneal epithelial cells that were exposed or not exposed to UVC irradiation were measured in the absence and presence of 4-aminopyridine (4-AP) by utilization of the Nikon Eclipse *Ti* (Nikon Instruments, Inc., Melville, NY, USA) inverted microscope with the following functions: (1) time-lapse videos of the phase-contrast/fluorescent live images, (2) built-in total internal reflection fluorescence (TIRF) and Förster resonance energy transfer (FRET); (3) perfect focus system (PFS), and (4) a digital charge-coupled device (CCD) camera in a time interval of 0.2 minutes for each photo. The system was equipped with a heated chamber at 37°C and flushed with mixed 5% CO_2 that kept cells in a normal culture condition. Live cells were recorded for a period of 0.5 to 3 hours. Cell motility was analyzed by tracking cell movements and distances (μm/h) using Nikon *Ti*'s NIS-Elements software.

Cell Volume Measurements

Changes in relative cell volume in the real time were estimated by measuring self-quenching of the fluorescent dye calcein.[36,37] Calcein-AM stock solution was made at a concentration of 10 μM in PBS with 1% DMSO. Corneal epithelial cells (70%–80% confluence) were loaded with calcein (200 μL per sample) and incubated at 37°C for 60 minutes. Unloaded calcein-AM is removed by washing wells three times with 1-mL warm (37°C) 1× PBS. Changes of calcein-AM intensity were measured using the Packard Fusion Microplate Analyzer (Conquer Scientific, San Diego, CA, USA) at 485- and 515-nm excitation and emission, respectively. Calcein-AM enters the cell and the AM-group is cleaved and the intracellular calcein becomes impermeable to the cell membrane. Thus, all the dye that has entered the cell is trapped within the cell. When the cell shrinks due to efflux of K^+ ions followed by water, the dye is concentrated in the cellular compartment. After the process of self-quenching, intensity of the dye decrease within the cell. The decrease in dye intensity is proportional to the decrease of the cellular volume.[36] The relative cell volume in isotonic solution was initially recorded for 5 minutes before UVC or hyperosmotic exposure. After UVC or hyperosmotic exposure, cell volume was measured every 0.1 to 0.5 minutes. In the measurement of UVC-induced volume changes, primary rabbit corneal epithelial cells were used because these cells were easier to be loaded with the dyes and less of leakages. In addition, both human and rabbit corneal epithelial cells had similar effects in responses to the UVC irradiation.

Western Blotting Experiments

Corneal epithelial cells (2×10^5) were lysed in sodium dodecyl sulfatepolyacrylamide (SDS) sample buffer containing 62.5 mM Tris-HCl pH 6.8, 2% wt/vol SDS, 10% glycerol, 50 mM DTT, 0.01% wt/vol bromophenol blue or phenol red. After denaturing, cell lysates were size-fractionated in 12% PAGE gels. Experimental samples in each testing tube are equally divided and loaded onto two identical protein gels that are running in the same gel apparatus, in the same condition and the same time. Proteins were electrotransferred to two identical polyvinylidene difluoride (PVDF) membranes (one
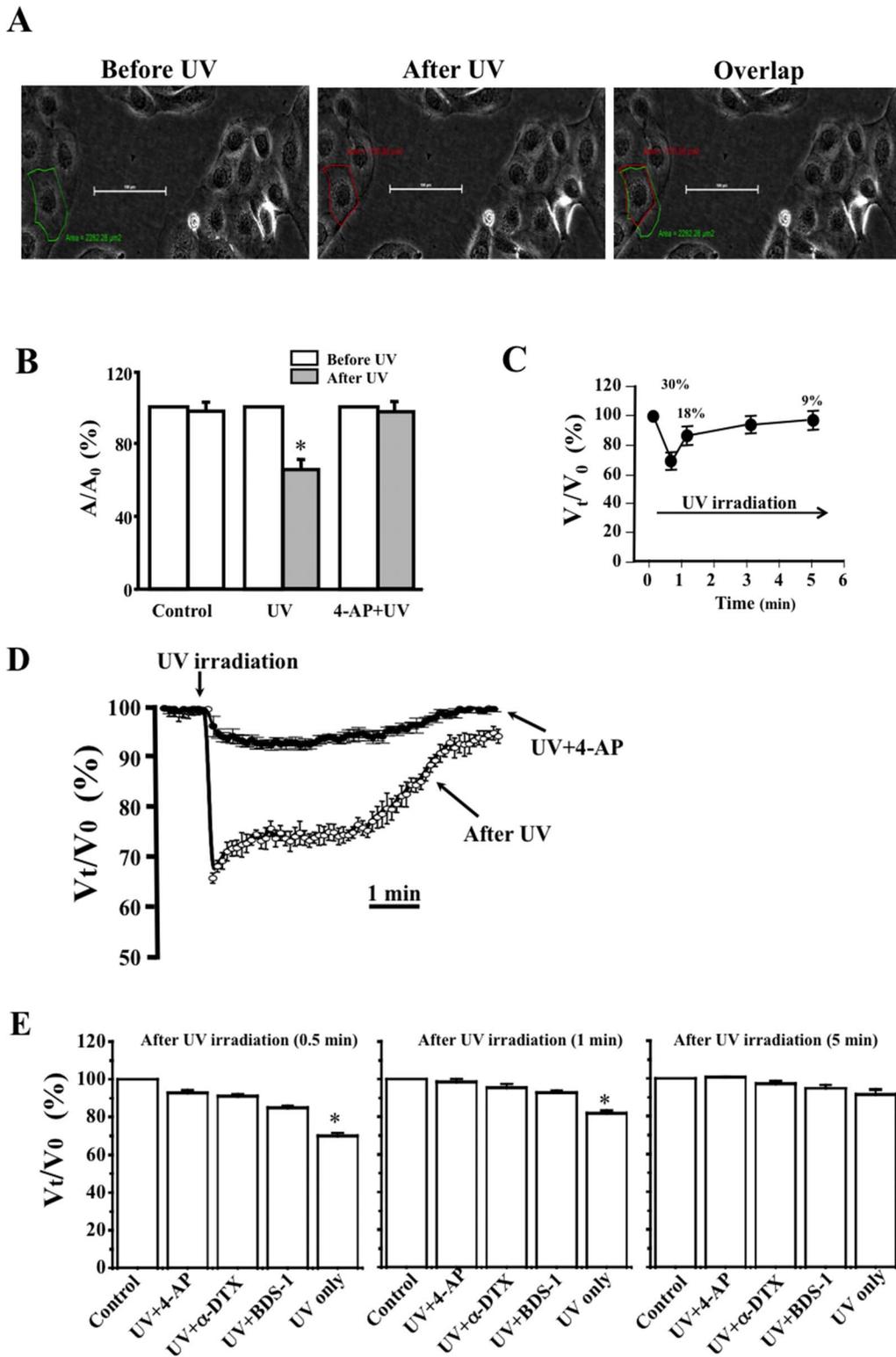
FIGURE 1. Effects of UVC irradiation on human and rabbit corneal epithelial cell sizes and volume decreases. (**A**) Ultraviolet C irradiation–induced cell size decrease recorded by a real-time video in human corneal epithelial cells. (**B**) Ultraviolet C irradiation–induced cell size decrease blocked by 4-AP in rabbit corneal epithelial cells. (**C**) Time course of UVC irradiation–induced cell volume changes in rabbit corneal epithelial cells. (**D**) Real-time recording of UVC-induced cell shrinkage. Rabbit corneal epithelial cells were exposed to UVC light in the absence and presence of 4-AP. (**E**) Suppression of UVC–induced cell volume decrease by various Kv channel blockers. The effect of Kv channel blockers on UVC-induced volume changes were measured at 0.5, 1, and 5 minutes after UVC exposure in the absence and presence of Kv channel blockers, including 0.5-mM 4-AP, 200-μM α-DTX, and 400-μM BDS-1. Ultraviolet C irradiation–untreated rabbit corneal epithelial cells were served as controls. Data were obtained from four independent experiments and plotted as means with *SE bars*. *Asterisks* represent the significant difference, $P < 0.01$.
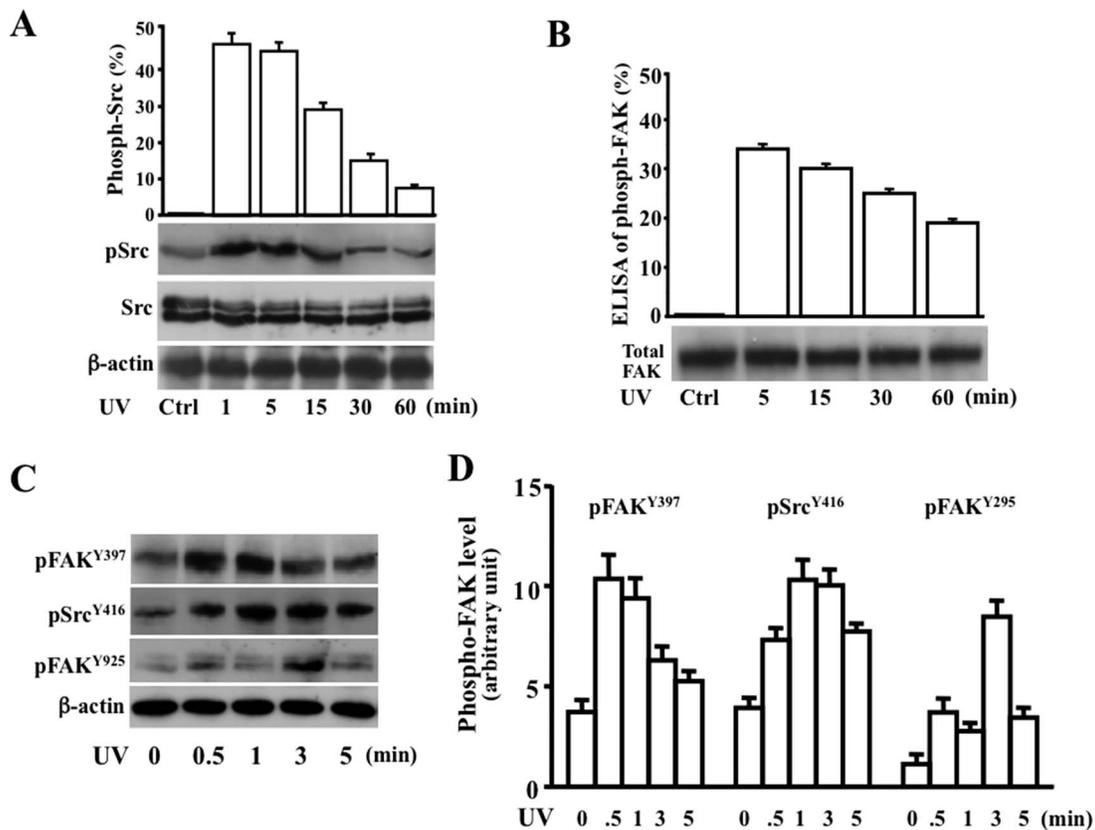
FIGURE 2. Effect of UVC irradiation on *Src* and FAK phosphorylation/activation in human corneal epithelial cells. (**A**) Ultraviolet C–induced changes of *Src* phosphorylation following a time course were detected by Western analysis. (**B**) Time course of UVC irradiation–induced FAK activation by detecting changes of FAK phosphorylation with ELISA. (**C**) Western analysis of UVC irradiation–induced site-specific phosphorylation of Src^{Y416}, FAK^{Y397}, and FAK^{Y925} following a time course. (**D**) Summarized time courses of site-specific phosphorylation of FAK in UVC irradiation–induced human corneal epithelial cells. These data demonstrate that UVC irradiation–induced cell volume alteration resulted in activation of *Src* and FAK signaling events.

of the membranes was for the loading control). They were exposed to blocking buffer containing 5% nonfat milk in TBS-0.1% Tween 20 (TBS-T) for 1 hour at 22°C, and then incubated with the primary antibodies at 4°C overnight. Horseradish peroxidase (HRP)-conjugated secondary antibody was applied in TBS-T buffer for 1 hour at 22°C. Western blots were developed by an ECL Plus System (Thermo Fisher Scientific, Inc., Santa Cruz, CA, USA) and visualized by exposure of X-ray films. Specific antibodies used in the study were phosphor-FAK Tyr-397 (antibody 3283), phosphor-FAK Tyr-925 (antibody 3284), and phospho-Scr Tyr-416 (antibody 2101) from Cell Signaling (Danvers, MA, USA).

Immunostaining and ELISA Experiments

For immunostaining experiments, corneal epithelial cells were grown on glass slides and treated as indicated in figures. The cells were fixed for 15 minutes in 4% paraformaldehyde, and then permeabilized with PBS-0.2% triton-X100 (PBS-T) for 30 minutes at 22°C. The tissues were blocked by incubation with 10% normal horse serum (NHS) or 10% normal goat serum in PBS-T for 1 hour at 22°C, followed by immunostaining with the corresponding antibodies, and/or incubated with FITC/Cy3-conjugated goat anti-mouse/rabbit IgG antibody (Cells Signaling). Corneal epithelial cells on slides were washed with PBS and stained with DAPI. A Nikon fluorescent Ti microscope was used to capture stained tissue imaging. Imaging data were analyzed using a Nikon NIS Element Software program. ELISA experiments were carried out using corresponding antibodies

against phospho-FAK and phospho-*Src* according to the company's instructions (Invitrogen). Briefly, corneal epithelial cells were exposed to UVC irradiation with/without adding Kv channel blockers in the indicated concentrations prior to lysis. The cell lysates were incubated in duplicate in the ELISA system.

Statistical Analysis

For Western analysis, signals in the films were scanned digitally and optical densities (OD) were quantified by using the Image Calculator software (Photometrics, Tucson, AZ, USA). The relative OD was calculated by normalizing the signals from target proteins against intensities of loading controls. The data of ELISA experiments were represented as the mean ± SD from three experiments independently performed in duplicates. All other experimental data were subjects to statistical analysis and plotted as mean ± SE. Significant differences between the control and treated groups were determined by 1-way ANOVA and Tukey's tests ($F < 0.05$). Student's *t*-test was used to determine the significant difference for paired samples at P less than 0.05.

RESULTS

UVC Stress–Induced Alterations of Cell Size and Volume

Changes in cell membrane K^+ channel activity can mediate functional adaptation to a variety of chemical and physical
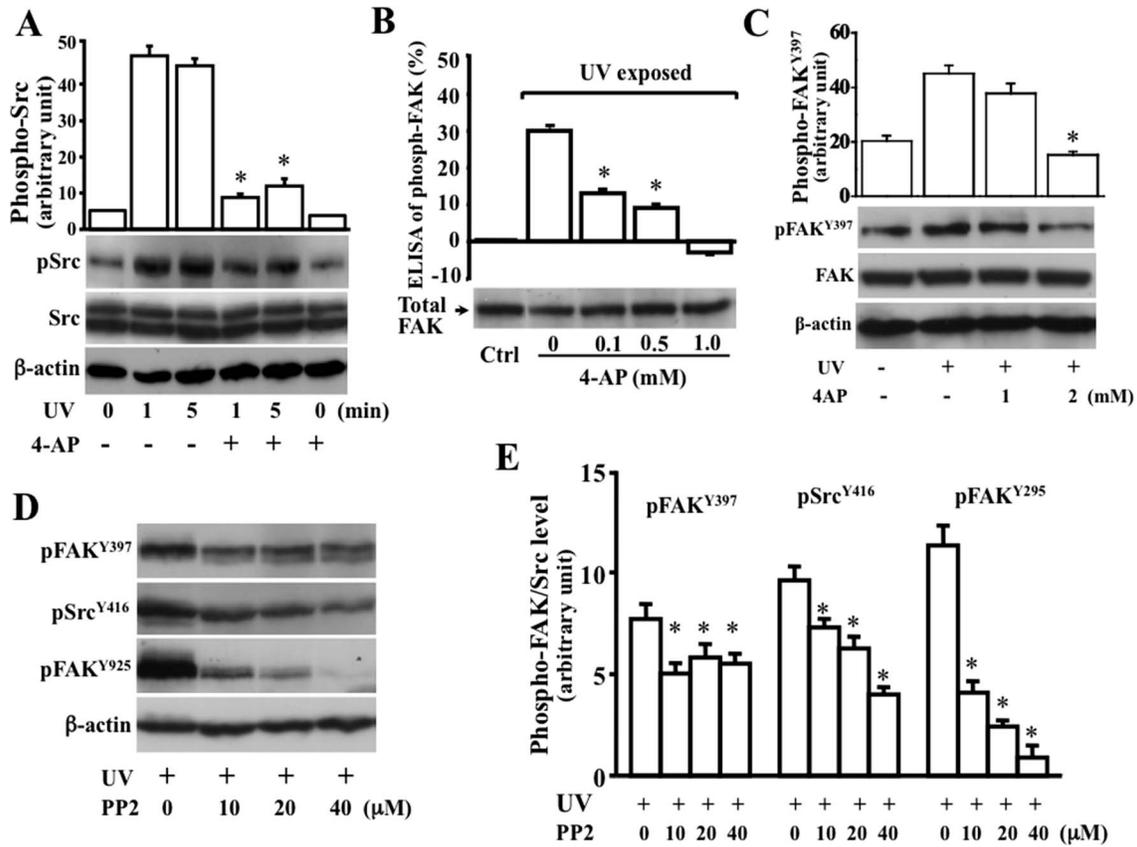
FIGURE 3. Effect of Kv channel blockade on *Src* and FAK phosphorylation/activation in human corneal epithelial cells. (**A**) Effect of blocking of Kv channel on UVC irradiation–induced *Src* phosphorylation/activation. Ultraviolet C irradiation–induced Kv channel activity in human corneal epithelial cells was blocked by 0.5-mM 4-AP and *Src* activation was detected by Western analysis. (**B**) Dose-response relationship of suppressing UVC irradiation–induced FAK activation by 4-AP. Human corneal epithelial cells were exposed to UVC irradiation in the absence and presence of various concentrations of 4-AP. Ultraviolet C irradiation–induced changes of FAK phosphorylation were determined by ELISA. (**C**) Effect of 4-AP on inhibition of UVC irradiation–induced FAKY397 phosphorylation detected by Western analysis. (**D**) Dose-response relationship of inhibiting UVC irradiation–induced *Src* and FAK site-specific phosphorylation by PP2. (**E**) Statistical analysis of various PP2 concentrations on site-specific inhibition of UVC irradiation-induced FAK phosphorylation. Effects of various concentrations of PP2 on phosphorylation levels of Src^{Y416}, FAK^{Y397}, and FAK^{Y925} in UVC irradiation–induced rabbit corneal epithelial cells were determined by Western analysis. Total cellular β–actin levels were detected as loading controls. Data are presented as means with *SE bars. Asterisks* represent the significant difference ($n = 4$), $P < 0.05$.

stresses through membrane voltage stabilization and maintenance of salt and water balance. Previously, we reported data from theoretical calculation and modeling, demonstrating that UV irradiation–induced hyperactivation of K^+ channels results in cell volume changes.[6] Ultraviolet C irradiation was applied to cultured human corneal epithelial cells. Alterations of the cell sizes were recorded as cell attached areas by real-time microscopy with a computerized head stage and cell tracking system. There was a remarkable change in cell size measured by areas of the attached cells after exposure to UVC irradiation (Fig. 1A). Statistical analysis showed that the measured cell areas were significantly changed before (A_0) and after (A) UVC irradiation (Fig. 1B). The effect of UVC irradiation on rabbit corneal epithelial cell volume decrease was measured in the absence (V_0) and presence (V_t) of UVC irradiation following a time course (Fig. 1C). The relative cell volume changes upon exposure of primary cultured rabbit corneal epithelial cells to UVC irradiation were plotted following a real-time measurement (Fig. 1D). The cell volume fell to the lowest point within 1 minute followed by a slow volume recovery. Ultraviolet C irradiation–induced rabbit corneal epithelial cell shrinkage was markedly inhibited in the presence of 4-AP, a Kv channel-specific blocker. In addition, the effect of suppressing Kv channel on UVC irradiation–evoked volume decrease was

further examined by adding a group of Kv channel blockers, including 4-AP (0.5 mM), α-dendrotoxin (α-DTX, 200 nM), and blood depressing substance-1 (BDS-1, 400 nM) at different time points (Fig. 1E). These results support the notion that UVC irradiation–induced cell size and volume decreases are resulted from hyperactivation of Kv channels resulting in a fast loss of intracellular K^+ ions in human and rabbit corneal epithelial cells, respectively.

Activation of FAK and Src Kinase Induced by UVC Irradiation

Ultraviolet C irradiation–induced FAK and *Src* activities were investigated in human corneal epithelial cells by measuring their phosphorylation levels using anti-phosphorylated *Src* and FAK antibodies, respectively. Total FAK and *Scr* levels were also detected as the loading controls. First, UVC irradiation–induced increase in phosphorylation *Scr* was detected by Western analysis following a 60-minutes time course (Fig. 2A). Ultraviolet C irradiation–induced change in FAK phosphorylation levels was detected by ELISA showing that the FAK phosphorylation level was significantly increased within 5 minutes and kept to be increased for 60 minutes after UVC exposure (Fig. 2B). Further studies were performed with
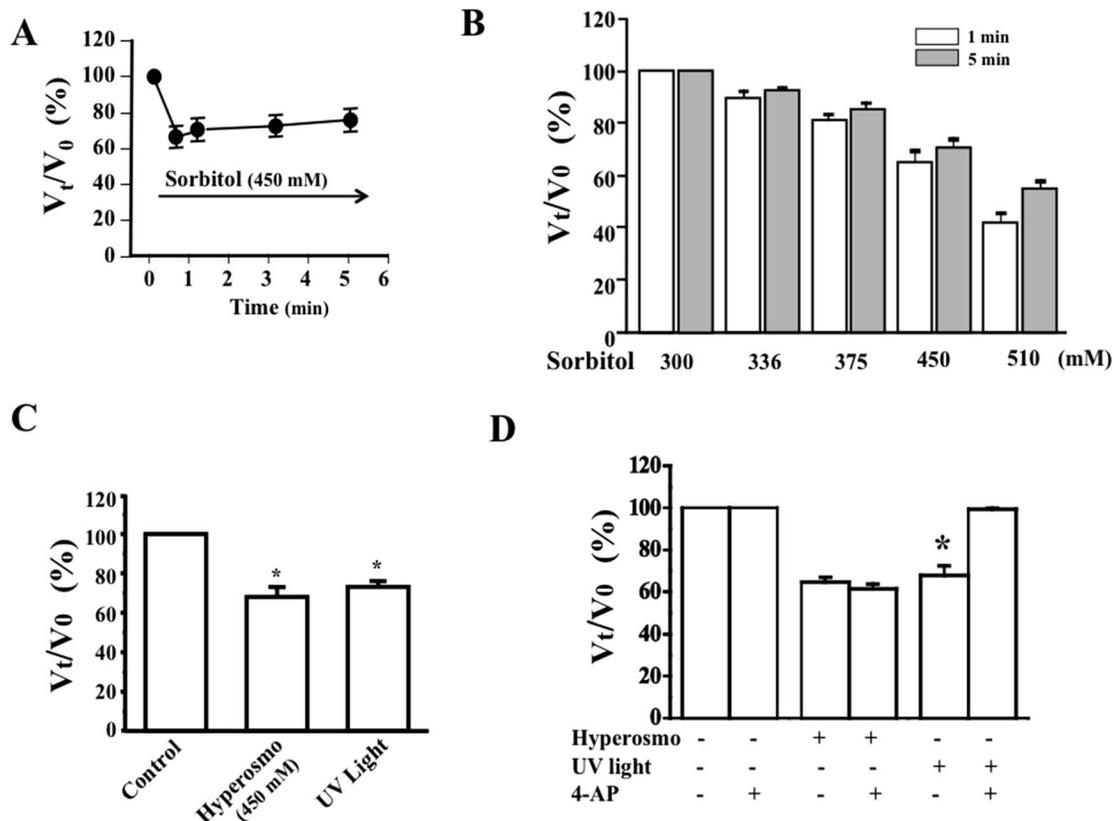
FIGURE 4. Effect of blocking Kv channel on hyperosmotic pressure–induced rabbit corneal epithelial cell volume changes. (**A**) Real-time recording of hyperosmotic pressure–induced changes in rabbit corneal epithelial cells. (**B**) Effect of various osmotic pressures by changing sorbitol concentrations rabbit corneal epithelial cell volume. (**C**) Comparison of cell volume changes in UVC irradiation– and hyperosmotic pressure (450 mM of sorbitol)–induced rabbit corneal epithelial cells. The time-points were selected for the peak time of cell volume changes for UVC exposure and hyperosmotic treatments. (**D**) Comparison of 4-AP effect on UVC irradiation– and hyperosmotic pressure–induced rabbit epithelial cell volume changes. Data are presented as means with *SE bars*. *Asterisks* represent the significant difference ($n = 4$), $P < 0.05$.

Western analysis by using anti-[Tyr(P)416]-*Src*, anti-[Tyr(P)397]-FAK, and anti-[Tyr(P)925]-FAK antibodies. Ultraviolet C irradiation–induced site-specific phosphorylations of active *Src* and FAK were detected and compared following a time course by using antibodies against phosphorylated forms of Src^{Y416}, FAK^{Y397}, and FAK^{Y925} (Fig. 2C). Statistical analysis showed that there were significant changes in phosphorylation levels of Src^{Y416}, FAK^{Y397}, and FAK^{Y925} following UVC irradiation (Fig. 2D). The results suggest that there is an early effect of UVC irradiation on phosphorylation of *Src* and FAK and that the relationship of the time courses between UVC irradiation–induced changes of the cell volume and phosphorylation of *Src* and FAK is closely correlated.

Effect of Kv Channel Blockade on UVC Irradiation–Induced Src and FAK Activation

We found that 4-AP effectively suppressed *Src* phosphorylation at 1 and 5 minutes after exposure of human corneal epithelial cells to UVC irradiation (Fig. 3A). Ultraviolet C irradiation–induced increases in FAK phosphorylation detected by ELISA were also effectively suppressed by 4-AP in a dose-dependent manner, which is in the same dosage range for 4-AP to block the UVC irradiation–induced volume decrease (Fig. 3B). The same site-specific FAK antibody against phospho-FAK^{Y397} was used in Western analysis to demonstrate activation of FAK induced by UVC irradiation in human corneal epithelial cells (Fig. 3C). Ultraviolet C irradiation–induced site-specific phosphorylation of FAK was effectively suppressed by 4-AP (2 mM).

Further experiments were performed by testing the effect of 4-amino-5-(4-chlorophenyl)-7-(t- butyl)pyrazolo[3,4-d] pyrimidine (PP2), a potent inhibitor of *Src* and FAK on site-specific phosphorylation of Src^{Y416}, FAK^{Y397} and FAK^{Y925}, following a dose-dependent pattern (Fig. 3D). There was a rather weak effect for PP2 to inhibit UVC irradiation–induced phosphorylation of FAK^{Y397}. Statistical analysis showed that PP2 significantly supressed UVC irradiation–induced phosphorylation of both Src^{Y416} and FAK^{Y925}. These results indicate that UVC-induced a site-specific phosphorylation of *Src* and FAK, resulting in activation of *Src* and FAK in UVC-exposed human corneal epithelial cells.

Comparison of Hyperosmotic and UVC Effects on Cell Volume

The effect of hyperosmotic pressure, established by adding 450-mM sorbitol in the medium, on human corneal epithelial cell volume decrease was also measured to compare with the UVC irradiation–induced volume decrease (Fig. 4A). There were continuous decreases in cell volume when osmotic condition in the extracellular medium was increased from the control level of 300 to 510 mM by adding various concentrations of sorbitol within 1 minute (Fig. 4B). Volume changes of the cells that were exposed to hypo-osmotic conditions were performed as the control measurements. Upon exposure of cells to 225- and 150-mM extracellular solutions, cell volume was increased approximately 25% and 75%, respectively (data not shown). After initial increase in the volume, the cell
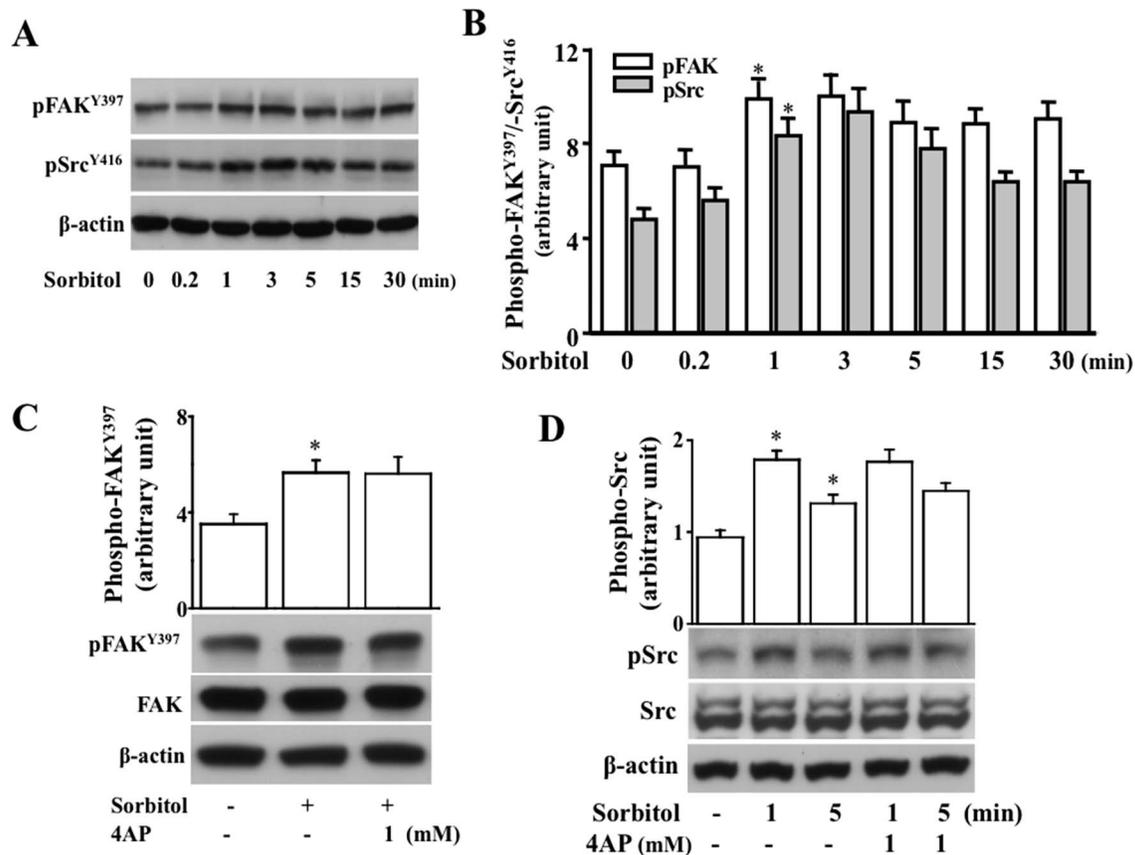
FIGURE 5. Effect of Kv channel blockade on hyperosmotic pressure–induced *Src* and FAK phosphorylation/activation in human corneal epithelial cells. **(A)** Time courses of hyperosmotic pressure–induced Src^{Y416} and FAK^{Y397} phosphorylation. **(B)** Statistical analysis of hyperosmotic pressure–induced Src^{Y416} and FAK^{Y397} phosphorylation. Effect of blockade of Kv channel on UVC irradiation–induced *Src* phosphorylation/activation. **(C)** Effect of 4-AP on hyperosmotic pressure–induced FAK^{Y397} phosphorylation in human corneal epithelial cells. **(D)** Effect of 4-AP on hyperosmotic pressure–induced Src^{Y426} phosphorylation in human corneal epithelial cells. Site-specific phosphorylation of *Src* and FAK was detected by Western analysis. Data are presented as means with *SE bars*. *Asterisks* represents the significant difference ($n = 4$), $P < 0.05$.

volume was gradually decreased toward the normal cell volume following a time course in the hypo-osmotic condition. In UVC irradiation–induced and hyperosmotic stress (450 mM)-stimulated cells, the peak volume changes were significant to show a volume shrinkage down to 67% and 65% in UVC irradiation–induced and hyperosmotic pressure (450 mM)-treated cells, respectively (Fig. 4C). However, applications of 4-AP had no significant effects on osmotic pressure–induced initial volume changes (Fig. 4D). The results reveal that UVC irradiation induced a significant cell shrinkage comparable to the effect resulted from hyperosmotic pressure stimulation.

Effect of Hyperosmotic Pressure–Induced Src and FAK Phosphorylation

The effect of hyperosmotic pressure on *Src* and FAK phosphorylation was measured in human corneal epithelial cells. Exposure of human corneal epithelial cells to hyperosmotic pressure resulted in increases in phosphorylation of Src^{Y416} and FAK^{Y397} following a time course (Fig. 5A). Statistical analysis showed the peak time of increased phosphorylation for FAK^{Y397} and Src^{Y416} at 1 and 15 minutes, respectively (Fig. 5B). Time courses of hyperosmotic pressure–induced activation of *Src* and FAK were similar to UVC irradiation–stimulated cells. Furthermore, application of K^+ channel blocker 4-AP at a dosage of 1 mM had no significant effects on hyperosmotic pressure–induced Src^{Y416} and FAK^{Y397} phosphorylation levels (Figs. 5C, 5D). In hyperosmotic

pressure-induced cells, both *Src* and FAK phosphorylation were increased as they have been observed in UVC irradiation–induced cell, suggesting that *Src* and FAK phosphorylation are cell volume change sensitive.

Immunostaining Detection of FAK Phosphorylation and Integrin β5 Clustering Induced by UVC Irradiation

Human corneal epithelial cells are attached cells. We predict that integrins in the edge area of the attached human corneal epithelial cells are affected by UVC irradiation–induced cell shrinkage. Activation of integrin β5 in UVC-irradiated cells was detected by immunostaining using the anti-integrin β5 antibody in the absence and presence of 4-AP. Integrin β5 formed clusters surrounding the edges of UVC irradiation–induced human corneal epithelial cells (Fig. 6A). The effect of UVC irradiation on integrin activation was effectively blocked by 4-AP. Further immunostaining experiments were performed to verify UVC irradiation–induced increases in phospho-FAK stained by using the antibody that specifically recognizes phospho-FAK^{Y397} (Fig. 6B). Cell nuclei were detected by using 4'6-diamidino-2-phenylindole (DAPI) staining. Ultraviolet C irradiation–induced increases of the phospho-FAK^{Y397} were effectively prevented by preincubation of cells with 4-AP. Blockades of Kv channel by 4-AP resulting in suppression of UVC irradiation–induced activations of integrin β5 and FAK
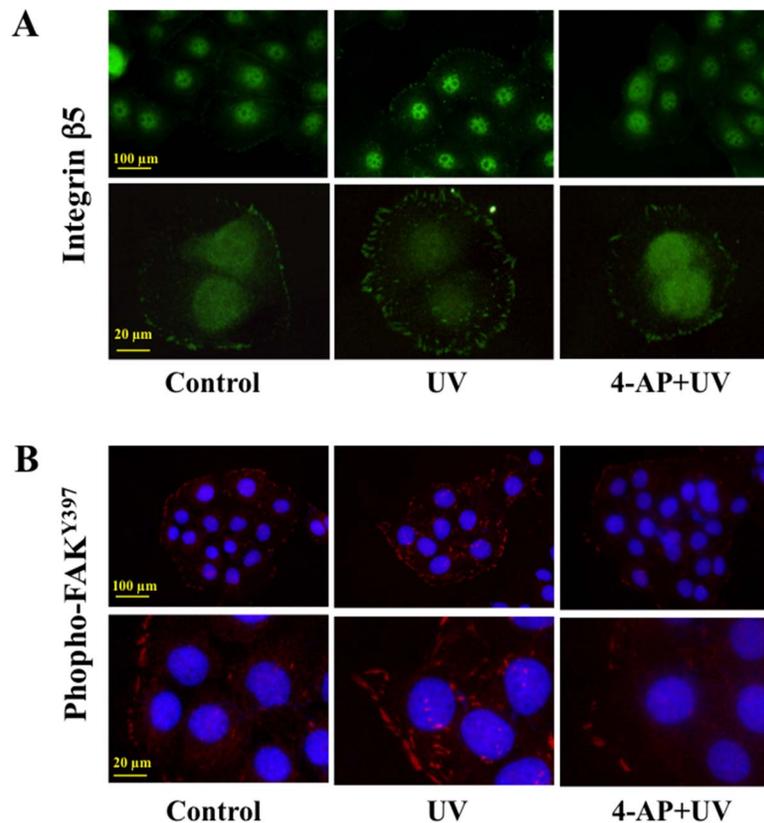
FIGURE 6. Immunostaining detection of UVC irradiation–induced alterations of integrin and FAK activities in human corneal epithelial cells. (**A**) Ultraviolet C irradiation–induced integrin β5 activation in the absence and presence of 4-AP in different scales. There were formations of integrin β5 clusters surrounding the edges of UVC irradiation–induced human corneal epithelial cells, which were blocked by 0.5-mM 4-AP. (**B**) Ultraviolet C irradiation–induced FAK[Y397] phosphorylation stained by red color and nuclei were stained with DAPI in the absence and presence of 0.5-mM 4-AP in different scales. Photos were taken by a Nikon fluorescent microscope (×20 and ×40).

provide further evidence that UVC irradiation–induced cellular changes are mediated by initial activation of K^{+} channels in UVC-exposed human corneal epithelial cells.

DISCUSSION

Environmental stresses, such as UVC irradiation, hyperosmotic pressure, hypoxia/reoxygenation, and infection, cause damages of corneal epithelia cells and activate various signaling pathways that delay the process of corneal epithelial self-renewal and wound healing. We have previously studied a mechanism involving UVC irradiation–induced hyperactivation of Kv channels and activation of MAP kinase signaling pathways in corneal epithelial cells.[7,11] Studies in various cell types suggest that the cell volume can be affected by activations of membrane ion channels resulting in significant increases in K^{+} efflux occurs prior to the volume change.[32] In addition, eliciting excessive K^{+} efflux or intracellular K^{+} depletion can result in apoptosis, which provides important evidence indicating that K^{+} channel activation plays a critical role to regulate cell fate.[38] However, it still lacks detailed mechanism that can explain how the cell shrinkage occurs in response to UVC-stress stimulation, and how the linkage between the stress effect and cell shrinkage leads to activation of signaling pathways, resulting in alteration of cell fate.

Results obtained from previous patch clamp studies in corneal epithelial cells provide essential information about loss of intracellular K^{+} ions resulted from UVC irradiation–induced hyper activation of K^{+} channels.[6] Theoretical calculation of cell volume based on our patch clamp data suggests that UVC irradiation–induced hyper activation of K^{+} channels in corneal epithelial cells result in a loss of nearly 3.6% of the total intracellular K^{+} stock per second.[6] If osmotic pressure change is considered, the change may be 10 mOsm per second, which equals to 3% of the intracellular osmolarity. The predicted volume decreases by the theoretical calculation is verified by the real-time measurement of the volume changes in UVC-exposed corneal epithelial cells, suggesting that UVC irradiation–induced K^{+} channel hyperactivation results in quick loss of intracellular K^{+} ions to decrease cell volume. In the present study, cell volume changes in corneal epithelial cells were measured in a real-time fashion with/without UVC irradiation by using a fluorescent dye calcein-AM. It has been shown that calcein-AM can be evenly distributed throughout the cell and used for the registration of volume alterations including epithelial cells.[36] We observed cell volume changes in a real time measurement, indicating a more than 25% decrease in cell volume in the first a few seconds of the UVC exposure. The degree of UVC irradiation–induced cell shrinkage is similar to the volume decrease in the cells exposed to 50% increased extracellular osmotic pressure. Ultraviolet C irradiation–induced cell volume alterations were effectively prevented by preincubation of cells with Kv channel-specific blockers, such as 4-AP, α-DTX, and BDS-1. These Kv channel blockers are effective in suppressing UVC irradiation–induced activation of MAP kinase pathways in corneal epithelial cells.[7,11] Taken together, shrinkages of UVC irradiation–induced corneal epithelial cells undergo through a fast time course. Thus,

UVC irradiation–induced activation of Kv channels in the membrane plays a key role in triggering cell volume changes.

Our previous studies in corneal epithelial cells have provided strong evidence showing that exposure of corneal epithelial cells to UVC irradiation induces the complex signaling events finally leading to alterations of the cell fate and wound healing.[7,11] The precise connections of UVC irradiation–induced ion channel activation, volume change and cytoskeleton reorganization have not been completely revealed. Here, we provide further evidence to demonstrate that exposure of corneal epithelial cells to UVC irradiation induces fast cell shrinkages. Blockade of Kv channel activity with specific blockers suppressed UVC irradiation–induced: (1) cell shrinkage, (2) activation of scaffolding protein kinase cascades, and (3) structural alterations of cytoskeletal proteins in corneal epithelial cells. The result suggests that changes of cell volume due to UVC irradiation–induced Kv channel hyperactivity activate the FAK-dependent integrin pathway that has been found to subsequently trigger cytoskeleton reorganization. Because corneal epithelial cells are attached cells, it is very likely that cell shrinkage in response to UVC irradiation results in a partial (or more precisely in the region of the cell edge) detachment of corneal epithelial cells evidenced by formations of integrin β5 clusters in UVC irradiation–induced cells. The leading edge of corneal epithelial cells is also the region where integrins first engage their ligands to activate the signaling pathways. Further downstream, activation of the FAK-dependent integrin signaling can also alter alignments of structural proteins, such as F-actin and α-tubulin. Structural changes of integrin and FAK phosphorylation were detected in UVC irradiation–induced cells by immunostaining using specific antibody against FAK^{Y397}. We found that UVC irradiation–induced integrin activation mainly in the edge of cells and formed a lot of clustering clouds in these areas, suggesting that UVC irradiation–induced cell volume changes may trigger alterations of the cytoskeletal structures. In addition, it has been shown that the FAK-dependent integrin-signaling pathway is activated during cell detachment and migration.

Apparently, UVC irradiation– and hyperosmotic stress–induced cell size and volume changes activate *Src* and FAK signaling events that can determine cell survival.[39,40] Phosphorylations of FAK and paxillin can be elicited in cultured cells by cyclical mechanical strain, and FAK couples integrins and cytoskeletal proteins to multiple signaling pathways. In addition, several lines of evidence suggest that FAK is required for stress stimulation to activate MAP kinase pathways including the JNK cascades.[41–45] These studies suggest that stress-induced cellular changes including cell shrinkage may play key roles in the early event in the initiation of FAK and *Src* activation, and cytoskeleton remodeling. Ultraviolet C irradiation–induced cell volume changes triggered activation of the FAK-dependent integrin signaling and cytoskeleton reorganization, critically determining UVC irradiation–induced early response in corneal epithelial cells. Previous studies show that UVC irradiation–induced hyperactivity of Kv channels in these cells,[7,11] blockade of KV channels with various Kv channel blockers in UVC-exposed cells significantly suppressed UVC irradiation–induced cell volume change, FAK/ *Src* activation, and cytoskeleton remodeling. Thus, a linkage between UVC irradiation–induced cell volume alteration due to loss of intracellular K$^+$ ions and activation of the FAK-dependent integrin pathway can be established in corneal epithelial cells. It also helps to explain the observation of cytoskeleton reorganization in response to UVC stress. Both scaffold kinases FAK and *Src* are important integrin receptor-linked tyrosine kinases to elicit cytoskeleton reorganization in many cell types. Our data using PP2 to suppress *Src* kinase activity is consistent with other lab's results that *Src* is responsible for further phosphorylation of FAK at different tyrosine sites after the site of Y397 is phosphorylated in these cells.[46] We found that K$^+$ channel blocker, 4-AP, effectively suppressed UVC irradiation–induced increases in FAK and *Src* phosphorylation or activation at micromolar levels, indicating that activations of FAK and *Src* are downstream events resulted from UVC irradiation–induced K$^+$ channel hyperactivity and cell shrinkage because their activities were altered by suppression of Kv channels in UVC irradiation–induced cells. We believe that the connection between UVC irradiation–induced activation of Kv channel and scaffold protein kinase in stress-evoked pathways plays important roles in determining corneal epithelial function and fate.

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