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Stress-induced corneal epithelial apoptosis mediated by K⁺ channel activation

Luo Lu^{*}

Department of Medicine, Division of Molecular Medicine, David Geffen School of Medicine, University of California Los Angeles, Harbor-UCLA Medical Center, 1124 W. Carson Street, C-2, Torrance, CA 90502, USA

Abstract

One of the functional roles of the corneal epithelial layer is to protect the cornea, lens and other underlying ocular structures from damages caused by environmental insults. It is important for corneal epithelial cells to maintain this function by undergoing continuous renewal through a dynamic process of wound healing. Previous studies in corneal epithelial cells have provided substantial evidence showing that environmental insults, such as ultraviolet (UV) irradiation and other biohazards, can induce stress-related cellular responses resulting in apoptosis and thus interrupt the dynamic process of wound healing. We found that UV irradiation-induced apoptotic effects in corneal epithelial cells are started by the hyperactivation of K⁺ channels in the cell membrane resulting in a fast loss of intracellular K⁺ ions. Recent studies provide further evidence indicating that these complex responses in corneal epithelial cells are resulted from the activation of stress-related signaling pathways mediated by K⁺ channel activity. The effect of UV irradiation on corneal epithelial cell fate shares common signaling mechanisms involving the activation of intracellular responses that are often activated by the stimulation of various cytokines. One piece of evidence for making this distinction is that at early times UV irradiation activates a Kv3.4 channel in corneal epithelial cells to elicit activation of c-Jun N-terminal kinase cascades and p53 activation leading to cell cycle arrest and apoptosis. The hypothetic model is that UV-induced potassium channel hyperactivity as an early event initiates fast cell shrinkages due to the loss of intracellular potassium, resulting in the activation of scaffolding protein kinases and cytoskeleton reorganizations. This review article presents important control mechanisms that determine Kv channel activity-mediated cellular responses in corneal epithelial cells, involving activation of stress-induced signaling pathways, arrests of cell cycle progression and/or induction of apoptosis.

1. Introduction

The corneal epithelium on the surface of the cornea plays both important and functional roles in the vision system (Lu et al., 2001b). For example, it forms a physical barrier with multiple layers of cells to prevent noxious agents from infecting and damaging the eye. These transparent cells, in conjunction with the ocular lens, are essential for appropriately refracting impinging light onto the retina and for maintaining normal optical properties of the vision. It is known that the preservation of the corneal epithelial function is dependent on the ability of the basal cells to proliferate at an adequate rate to replace the dying cells in the superficial layers. Corneal epithelial cells in the basal layer proliferate continuously to replace the superficial cell layers that slough off and are carried away by tears. The maintenance of corneal epithelial cells in healthy and functional conditions is a dynamic process to ensure normal corneal optical properties when the tissue undergoes continuous renewal.

*Tel.: +1310 787 6853; fax: +1310 222 3781. E-mail address: llou@ucla.edu.

Ion channels play central roles in maintaining ion and fluid balance in order to dehydrate the cornea and to prevent corneal swelling. The dehydration process in the cornea is important for maintaining corneal transparency (Dikstein and Maurice, 1972; Maurice, 1972). These attributes are dependent on the function of ion channels and ion transports that generate osmotic gradients (the driving force) for fluid flows. The driving force provides the ability of the outer epithelial and inner endothelial limiting layers to elicit fluid flows towards the tears and the anterior chamber. Such movement offsets stroma fluid imbibition, which if left unchecked, leads to corneal swelling and translucence. In the human cornea, the monolayer and essentially non-divided endothelial cells in the inner cornea play a major role in maintaining corneal optical properties by removing fluids out of the cornea (Maurice, 1972). It elicits net fluid transport outward from the stroma into the anterior chamber, which offsets the natural tendency of the stromal ground substance e.g., proteoglycans to imbibe fluid and swell. The corneal epithelial layer provides a partial fraction of the total dehydrating capacity resulting from osmotically coupled fluid flow. Under maximally stimulated conditions, it is estimated that the epithelium contributes about 25% of the total dehydrating function of the limiting epithelial and endothelial layers (Klyce, 1977). With normal functioning ion channels and transporters, the dehydration process is sustained to keep constant cell volume and to provide the integrity of tight junction between neighboring epithelial cells. Tight junctions between neighboring epithelial cells provide a highly resistant junctional barrier with corneal epithelial cells. The integrity of the tight junction is needed for the corneal epithelial barrier function (Klyce, 1972). It has been demonstrated that corneal epithelial renewal is a continuous process, which is essential for wound healing in the cornea. Wounding of the corneal epithelial surface leads to breakdown of tight junctional integrity due to the loss of the outer limiting epithelial layer. This loss can result in breakdown of cell membrane permeability and selectivity. The damaged integrity is not restored until after epithelial migration from the periphery. A possible outcome of wounding and loss of tight junctional integrity is that the corneal interior becomes vulnerable to infection by invasive pathogens. Such a scenario could also lead to the development of corneal opacity as the result of decreases in endothelial fluid transport, which could increase stromal hydration.

During the development of cells, ubiquitous ion channels sense chemical and physical changes in the cell environment and mediate the functional adaptation of cells to environmental changes. The voltage-gated K^+ channels are widely distributed and involve maintaining electrophysiological stabilization which is essential for salt and water balance in kidney cells (Wang et al., 1992) and guard cells (Schroeder et al., 1994), cell volume regulations (Deutsch and Chen, 1993), electrical excitability in neurons and cardiac muscle (Jan and Jan, 1989), insulin release from pancreatic B-cells (Dukes and Philipson, 1996), activation of T- and B-lymphocytes (DeCoursey et al., 1984; Amigorena et al., 1990b), and controlling myeloblastic cell development (Lu et al., 1993). More emerging evidence suggest that K^+ channels play a crucial role in proliferation control in different cell types including corneal epithelial cells (DeCoursey et al., 1987; Freedman et al., 1992; Day et al., 1993; Deutsch and Chen, 1993; Pappone and Ortiz-Miranda, 1993; Xu et al., 1996, 1999; Wang et al., 1997; Roderick et al., 2003). The activation of K^+ channels is involved in the onset of cellular events associated with both T- and B-lymphocyte activation (Amigorena et al., 1990a,b; Lin et al., 1993). Enhanced K^+ channel expression or K^+ channel activity is associated with mitogenesis in several cell types (DeCoursey et al., 1984; Price et al., 1989). Suppression of K^+ channel activity by blockers in culture significantly inhibits cell proliferation (Deterre et al., 1981; Amigorena et al., 1990b; Dubois and Rouzaire-Dubois, 1993; Lin et al., 1993). K^+ channel activity has been found to be a key determinant for cell progression through the G_1 checkpoint of the cell cycle (Lewis and Cahalan, 1990; Dubois and Rouzaire-Dubois, 1993; Xu et al., 1996; Roderick et al., 2003). In K^+ channel activity-suppressed cells, retinoblastoma protein (pRB) is dephosphorylated and effectively inhibits the cell from progressing through the G_1/S transition (Xu et al., 1996; Roderick et al., 2003; Wang J et al., 2004). These results suggest that changes

in K⁺ channel activity can modulate the magnitude of a mitogenic response to a growth factor in different cell types, including in corneal epithelial cells.

Recent studies demonstrate that drastically changed activities of K⁺ channels are most likely involved in mediating programmed cell death (apoptosis) because some apoptosis-inducing factors such as reactive oxygen species (ROS), ultraviolet (UV) irradiation, tumor necrosis factor (TNF) and anticancer drugs can significantly alter K⁺ channel activity. (Soliven et al., 1991; Dubois and Rouzaire-Dubois, 1993; Bright et al., 1994; Duprat et al., 1995; Szabo et al., 1996; Wang et al., 1999a,b; Lu et al., 2003; Gao et al., 2004; Guo et al., 2005). For example, we, as well as other researchers, have shown that stimulation of K⁺ channel activity and dramatic loss of intracellular K⁺ can result in apoptosis in the corneal epithelium, neurons, myeloblastic ML-1 cells, and other cell types (Schulz et al., 1996; Eldadah et al., 1997; Yu et al., 1997; Wang et al., 1999a,2003; Lu et al., 2003; Trimarchi et al., 2002; Remillard and Yuan, 2004). It seems that there are dual functions of K⁺ channels involving cell proliferation regulated by epidermal growth factor (EGF) (Lu et al., 2001a) and playing a role in mediating cell death induced by UV irradiation (Wang et al., 1999a,2003; Lu et al., 2003). We found in corneal epithelial cells that K⁺ channel activity is required for growth factor-stimulated activation of extracellular-regulated kinase (ERK) (a MAP kinase) limb and proliferation. Hyperactivation of K⁺ channels by UV irradiation can cause a rapid and mass loss of intracellular K⁺ ions resulting in activation of c-Jun N-terminal kinase (JNK) limb; increase in p53 phosphorylation and promoting apoptosis.

2. Subtypes of K⁺ channels found in corneal epithelial cells

2.1. Expression of Kv channels in corneal epithelial cells

It is important to identify the type of K⁺ channels in the corneal epithelium that are involved in mediating mitogenic responses and apoptosis induced by growth factors, cytokines and stress stimulation, respectively. In rabbit and human corneal epithelial cells, there are voltage-gated K⁺ (Kv) channels in the apical membrane of the superficial layers and a large conductance K⁺ channel is also found in the basal cell layers (Rae, 1985; Rae et al., 1990; Rae and Farrugia, 1992). These channels are stimulated by fenamate, cGMP, carbachol, cell swelling, membrane stretch, CO and acidification (Farrugia and Rae, 1992,1993; Rae et al., 1992; Rae and Farrugia, 1992; Rich et al., 1994,1997; Watanabe et al., 1997). Their activities are inhibited by barium, quinidine, diltiazem and prozac (Rae and Farrugia, 1992; Rae et al., 1995). The whole-cell currents were further characterized in cultured human corneal epithelial cells (Bockman et al., 1998). In these cells, there is a depolarization-activated outward rectifying K⁺ current, a hyperpolarization-activated outward rectifying K⁺ current, and an inward rectifying K⁺ current (Bockman et al., 1998). Recently, the mRNA of the inward rectifying Kv (Kir2.1) channel has been reported in this tissue (Rae and Shepard, 2000). The bovine corneal epithelium exhibits two types of outward K⁺ current: an inactivating voltage-gated K⁺ current which is inhibited by arachidonic acid, and a noisy and sustained K⁺ current (Takahira et al., 2001). Our previous results indicated that there are voltage-gated and 4-aminopyridine (4-AP)-sensitive K⁺ channels in rabbit and human corneal epithelial cells. However, the specific subfamily members contributing to the K⁺ currents have yet to be completely evaluated in corneal epithelial cells.

It is undeniable that several K⁺ channel subtypes are expressed in corneal epithelial cells because previous studies showed that changes in K⁺ channel activity modulate essential corneal epithelial functions needed for tissue homeostasis (Klyce and Wong, 1977; Wolosin and Candia, 1987). As shown in these studies, such changes are essential for mediating changes in net ion transport, cell cycle progression and apoptosis resulting from exposure to cAMP mobilizing agonists, growth factors and UV light, respectively. Each of these responses is dependent on K⁺ channel activation. Therefore, corneal epithelial renewal, which is a result of

a balance between cell proliferation, apoptosis and differentiation, is dependent on K⁺ channel activation by stimuli that affect each of these responses (Lu et al., 2003; Wang et al., 2003). There is one type of K⁺ channels in corneal epithelial cells with characteristics similar to Kv3.4 channels. Kv3.4 channels belong to the mammalian shaker-related K⁺ channels and are a member of the *Shaw* subfamily (Kanemasa et al., 1995). These channels exhibit relatively fast inactivation as do the Kv1.4, Kv4.1, Kv4.2, and Kv4.3 channels. Kv3.4 expression has been found in the hippocampus, cerebellum, brain stem, spinal cord, skeletal muscle, arterial smooth muscle cells and pancreatic acinar cells (Weiser et al., 1994; Vullhorst et al., 1998; Rudy et al., 1999; Gopel et al., 2000; Abbott et al., 2001; Han et al., 2002; Martina et al., 2003). It performs an important role in modulating electrical excitability of neurons and muscle fibers. Prior to our report, there is no report about gene expression and functional studies of Kv3.4 channels in the corneal epithelium. Kv3.4 is one of the voltage-gated and 4-AP sensitive K⁺ channels existing in human, rabbit and rat corneal epithelial cells. Given the importance of K⁺ channel modulation in the control of epithelial renewal, identification of Kv3.4 expression provides important information for possible drug targets that can be used to develop new specific Kv3.4 modulators for use of stimulating the epithelial renewal process.

2.2. Kv3.4 channel in corneal epithelial cells

Approaches used to characterize the specific Kv channel subfamily expressed in corneal epithelial cells include immunohistochemistry, Western analysis, voltage- and patch-clamp. *First*, expression of the Kv channels and their distributions were screened and evaluated by using immunohistochemistry methods, respectively. After screening with a panel of selective Kv channel antibodies, specific Kv3.4 expressions were detected and delimited in the plasma membrane by immunostaining experiments. Cell membrane localization of the Kv channel was further confirmed with confocal microscopy showing that there is indeed punctate labeling in the cell membrane (Fig. 1A and B). Furthermore, such punctate labels were more intense in the basal proliferating layer, which is consistent with the known importance of K⁺ channels in mediating mitogenic responses by this cell layer in response to growth factors (Roderick et al., 2003; Li and Lu, 2005). Control experiments were performed by pre-absorption of the anti-Kv3.4 antibody with the antigenic peptide to block the immunocytochemical response and by omission of the primary antibody. Both control experiments resulted in no stains that validate the specificity of the staining reactions. Another line of evidence supporting Kv3.4 expression in localization of the plasma membrane is that the Western blot analysis reveals Kv3.4 channel proteins presence in the corneal epithelium in both whole-cell protein preparation and enriched plasma membrane fractionation, but Kv3.1, Kv4.2 or Kv4.3 channel proteins were not detected with these methods (Fig. 1C). *Second*, patch-clamp experiments demonstrate that the whole-cell K⁺ current in rabbit corneal epithelial cells is voltage-dependent with a fast activation and a gradual inactivation (Wang L et al., 2004). The pharmacological profile of the whole-cell K⁺ current in rabbit corneal epithelial cells is similar to that described for the Kv3.4 channel. Both 4-AP (100 μ M) and α -dendrotoxin (α -DTX) (200 nM) effectively inhibited whole-cell K⁺ currents, indicating that the Kv channel in these cells is Kv3.4 (Fig. 2A). Additional evidence from blood depressing substance-I (BDS-I) experiments provide further support to this notion because BDS-I, a highly specific blocker of Kv3.4 channel, does not block other types of Kv channels, such as Kv1.4, Kv2.1, and Kv4.1–Kv4.3 (Diochot et al., 1998). BDS-I significantly reduced Kv currents in rabbit corneal epithelial cells confirming that the Kv channel in corneal epithelial cells indeed belongs to Kv3.4 subfamily (Fig. 2B). In addition, electrophysiological characterizations of the whole-cell Kv currents reveal that the activation pattern of the whole-cell K⁺ current in corneal epithelial cells is similar to those described for Kv3.4 channels in the brain and in the skeletal muscle, in which the activation phase is fast. However, the inactivation phase of the whole-cell K⁺ current in corneal epithelial cells is much slower than the Kv3.4 channels found in excitable tissues. A recent report of a voltage-gated K⁺ channel in bovine corneal epithelial cells possesses a similar pattern as the Kv3.4 current

in RCE cells (Takahira et al., 2001). The possible explanation for the difference of Kv channels in corneal epithelial cells compared to those found in excitable tissues is probably that Kv channels serve different functions in these tissues. Instead, Kv3.4 channels in corneal epithelial cells may function as mediators to elicit activation of a host of different signaling pathways that are needed for the control of cell cycle progression, apoptosis and modulation of net ion transport.

2.3. Activation of K⁺ channels by cytokines in corneal epithelial cells

Activation of K⁺ channels by growth factors/cytokines has been found in many cell types. It has been shown that nerve growth factor (NGF) regulates the abundance and distribution of delayed rectifier K⁺ channels in PC12 cells (Sharma and Lombroso, 1995). Exposure of microglial cells to interferon- γ (IFN- γ) or granulocyte/macrophage-colony stimulating factor (GM-CSF) results in an enhancement of outward K⁺ current (Fischer et al., 1995). These results suggest that a shift of the resting membrane potential towards more hyperpolarized levels may be a prerequisite for intracellular activation of the proliferative response in macrophage and microglial cells. In cultured human oligodendrocytes, inward rectifier K⁺ channels were modulated by TNF- α , a cytokine associated with activated macrophages (Soliven et al., 1991; McLarnon et al., 1993). Studies from our lab and others have demonstrated that K⁺ channel activity is linked to proliferation and apoptosis in corneal epithelial cells and in other various systems (Lu et al., 1993; Mauro et al., 1993; Xu et al., 1996). Recent studies show that K⁺ channel activity in corneal epithelial cells and other cell types can be modulated by a variety of growth factors and cytokines (Lu et al., 1993; Wilson et al., 1993; Fischer et al., 1995; Sharma and Lombroso, 1995; Wang et al., 1997). In general, effects of growth factors and cytokines on K⁺ channel activity can be divided into short-term and long-term effects. The short-term effect is characterized by the alteration of channel gating, most likely through a second messenger system-mediated modulation of the channel protein. However, the long-term effect corresponds to a maximal increase in the whole-cell K⁺ conductance resulting from increases in total channel numbers. This can be caused by changes in K⁺ channel genes and/or protein expression levels and the rate of membrane insertions. However, the short-term effect of growth factors and cytokines on K⁺ channel activity may play the major role in regulation of cell proliferation and apoptosis.

2.4. EGF-induced K⁺ channel activity

In recent studies, we showed that EGF and fetal bovine serum (FBS) induce increases in proliferation by mediating rises in whole-cell currents and a selective conductance of 31 pS K⁺ channel (Fig. 3A). In comparison with corneal epithelial cells cultured in serum-starved conditions for 24 h, K⁺ channel activity at a membrane potential of -60 mV was very low. Application of FBS or EGF in the patch chamber rapidly stimulated the single-channel and whole-cell K⁺ channel currents by using cell-attached single channel and nystatin-perforated whole-cell patch clamping, respectively. In the single channel level, application of 5 ng/ml EGF following serum starvation increased K⁺ channel activity within 10–20 min (Fig. 3B). K⁺ channel activity at -60 mV significantly increases from 2.0 $\pm$ 0.4% to 38 $\pm$ 4% within 20 min, comparable to those observed in medium containing 10% FBS in which K⁺ channel activity significantly increases from 2.0 $\pm$ 0.4% to 40 $\pm$ 4% within 20 min. The other evidence that supports an association between K⁺ channel activity and mitogenesis is that endothelin-1 (ET-1), a weaker mitogen than EGF, can stimulate increase in K⁺ channel activity. Application of 2 nM ET-1 continuously increases K⁺ channel activity although ET is much less effective than EGF in rabbit corneal epithelial cells. When combination of EGF and ET-1 is used in these cells, it demonstrates additive effects of stimulation on K⁺ channel activity. This suggests that an increase in K⁺ channel activity is associated with the stimulation of proliferation because serum addition stimulates K⁺ channel activity more than either EGF or ET-1. The additive effects of EGF and ET-1 on K⁺ channel activity are possibly due to different signaling pathways

stimulating K⁺ channel activity. The physiological significance of the growth factor-induced stimulation of proliferation is related to its heightening of K⁺ channel activity, which results in membrane potential hyperpolarization. As in corneal epithelial cells, an increase in calcium influx may be the result of an increase in membrane potential hyperpolarization, which is consistent with our finding that EGF induces an increase in K⁺ channel activity. In fact, membrane potential hyperpolarization may increase calcium entry through capacitative calcium channels that have been found in rabbit corneal epithelial cells as a component of signaling pathways linked to EGF receptor stimulation (Yang et al., 2003). Thus, K⁺ channel activation and membrane potential hyperpolarization increase in calcium influx may also play a role in EGF-induced stimulation of proliferation. This is apparent because pharmacological inhibition of neither K⁺ channel nor calcium influx activities also blocks EGF-induced stimulation of proliferation (Wang et al., 1997; Yang et al., 2003). More importantly, EGF stimulates RCE cell proliferation through activation of ERK (MAP kinase) signaling cascades (Kang et al., 2000, 2001). The ability of EGF to activate the ERK pathway is also dependent upon its ability to activate K⁺ channel activity. In previous work, we have shown that the activation of a voltage-gated K⁺ channel in hematopoietic ML-1 cells by EGF stimulation promotes cell proliferation. Suppression of K⁺ channel activity inhibits cell proliferation through inhibition of extracellular-regulated protein kinase 2 (Erk-2) signaling pathway (Xu et al., 1999). A difference from RCE cells, this inhibition of Erk-2 activation is independent of extracellular Ca²⁺, indicating that K⁺ channel does not affect Erk-2 activity by regulating Ca²⁺ influx. In addition, activity of the K⁺ channels is required for multiple types of cells including RCE cells to progress through the G₁ phase of the cell cycle (Wilson et al., 1996; Wonderlin and Strobl, 1996; Wang et al., 1997). Suppression of the K⁺ channels with specific K⁺ channel blockers inhibits cell proliferation by preventing the G₁/S transition of the cell cycle, suggesting that the activity of K⁺ channels is required for the early events of the cell proliferation process (Xu et al., 1996).

2.5. K⁺ channel activity evoked by TNF- α

TNF- α has pleiotropic effects in many cell types that include activation of apoptosis, inflammation and immune responses. TNF- α is also one of a number of cytokines found in tears and its expression is elevated in allergic conjunctivitis (Cook et al., 2001). TNF- α is secreted by corneal epithelial cells in response to an inflammatory stimulation caused by bacterial and viral infections (Minami et al., 2002; Kurpakus-Wheater et al., 2003; Bitko et al., 2004; Kumar et al., 2004). TNF- α -stimulated increases in K⁺ channel activities are important for TNF- α -induced cellular effects in cortical neurons, kidney epithelial cells, hepatic cells and corneal epithelial cells (Houzen et al., 1997; Nietsch et al., 2000; Wei et al., 2003). TNF- α also induces mRNA expression of various K⁺ channel types during a systemic inflammatory response as well as tumor cell proliferation in brain and cancer cells (Wang et al., 2002; Vicente et al., 2003, 2004). For example, treatment of oligodendrocytes with TNF- α for 24–48 h significantly decreases expression of the K⁺ channel gene and reduces the mean open time of the K⁺ channel relative to control values. These data suggest that TNF- α exerts both short- and long-term effects on the inward rectifier K⁺ channel in human oligodendrocytes. However, TNF- α suppresses particular types of K⁺ channel activity in other cell types, indicating that the effect of TNF- α on K⁺ channel activity is dependent on the channel type and cell origins (Vicente et al., 2004; Wang J et al., 2004). In corneal epithelial cells, TNF- α does not induce apoptosis, but instead it increases the expression of p21 resulting in cell cycle attenuation in the G₁ phase (Wang et al., 2005). We, as well as others, in numerous earlier studies have shown that K⁺ channel activity is the major conductance in the cell membrane. TNF- α induces increases in activity of a 4-AP-sensitive K⁺ channel in human corneal epithelial cells. The effects of TNF- α (20 ng/ml) on K⁺ channel activity were recorded using the cell attached patch clamp (Fig. 4A). In serum-starved cells, robust K⁺ channel activity was elicited upon application of TNF- α onto the patched chamber at a membrane potential of –60 mV. TNF- α -

induced K⁺ channel activities were not run-down up to 30 min of recording time. The channel opening properties are analyzed and found to be significantly increased to $30.2 \pm 3.1\%$ ($n = 8$), compared to K⁺ channel activity in control cells ($9.1 \pm 1.6\%$, $n = 14$). Application of 1 mM 4-AP in the patch pipette markedly suppressed TNF- α -induced K⁺ channel activity resulting in the channel opening to decline to $8.4 \pm 0.8\%$ (Fig. 4B). As mentioned above, TNF- α -induced attenuation of cell cycle progression results in a rise in the cell population in the G₁ phase. The effect of TNF- α on cell cycle progression is associated with an increase in cell viability. Accordingly, the dependence of TNF- α -induced cell cycle attenuation on K⁺ channel activity is studied by evaluating the viability of cells stimulated with 20 ng/ml TNF- α in the absence and presence of various dosages of 4-AP. In these experiments, corneal epithelial cell death is determined by detection of chromatin condensation using nuclear staining with ethidium bromide (EB) and acridine orange (AO). Cell death in response to TNF- α (20 ng/ml) or 4-AP (1 mM) does not occur. However, suppression of K⁺ channel activity with 4-AP in TNF- α -induced cells triggered 4-AP dose-dependent programmed cell death (Fig. 4C). This suggests that TNF- α promotion of cell survival is dependent on its ability to induce increases in K⁺ channel activity. TNF- α may activate other pathways besides K⁺ channel pathways. The biophysical property of the TNF- α -induced K⁺ channel resembles a Kv3.4 channel that is consistent to the previous identification of the channel using a specific panel of K⁺ channel antibodies (Wang L et al., 2004). Such stimulation elicits activation through possibly a myriad of changes that include initially volume shrinkage leading to changes in cytoskeletal configuration and scaffolding protein conformation. TNF- α -induced increases in K⁺ channel activity is probably upstream from activation and nuclear translocation of NF κ B because exposure to 200 μ M 4-AP prevents TNF- α -induced NF κ B binding to DNA and its nuclear translocation. There is a good agreement between the inhibitory effect of 4-AP on K⁺ channel activity and those on NF κ B activation because previous studies indicate that 4-AP inhibits K⁺ channel activity also at μ M levels dependent on the membrane potential (Wang et al., 2003; Wang L et al., 2004). Suppression of K⁺ channel activity obviates the protective effect of TNF- α on cell death. Overriding this protective effect results in an increase in apoptosis of corneal epithelial cells, while application of TNF- α or 4-AP alone to cells does not induce apoptosis. These results further indicate that this cell membrane channel activity is responsible for initiation of TNF- α -induced activation of NF κ B signaling that is essential for cell survival in response to cytokine and stress stimulations. In corneal epithelial cells, UV irradiation can induce hyper-activation of K⁺ channels with a magnitude of 2-fold greater than that of mitogen-induced channel activity (see next section). The hyper-activation of K⁺ channels at this magnitude may cause an excessive loss of intracellular K⁺ ions, resulting in a rapid cell shrinkage, activation of JNK cascades and other catastrophic cellular effects. Their diverse control suggests that there exists crosstalk at various levels between K⁺ channel activity in the cell membrane and growth factor receptor-linked signaling cascades or stress-induced signaling pathways. However, the exact details about these effects cannot be dealt with here because it is a topic for future investigation. In summary, TNF- α -induced stimulation of a 4-AP-sensitive K⁺ channel is required for NF κ B nuclear translocation and DNA binding activity, which in turn promotes cell survival. K⁺ channels are universally present in the cell membrane and important in maintaining growth factor-stimulated cell growth and UV irradiation-induced apoptosis.

2.6. UV irradiation-induced K⁺ channel hyperactivity

There are numerous reports that concern the high susceptibility of corneal epithelial cells to damages caused by UV irradiation (Guymer and Mandel, 1989; Cejkova and Lojda, 1994; Kolozsvari et al., 2002; Cejkova et al., 2004). However, there is currently very little information regarding how UV irradiation induces corneal epithelial cell apoptosis (Lu et al., 2001b). Recent studies provide cellular and molecular mechanisms involving hyperactivation of a K⁺ channel in corneal epithelial cells (Lu et al., 2003; Wang et al., 2003). Data from earlier

studies of our lab and others show that blocking K^+ efflux by a K^+ channel blocker or by increasing extracellular K^+ inhibited apoptosis in human eosinophil, human myeloid cells and mouse cortical neurons (McCarthy et al., 1994; Beauvais et al., 1995b; Kaneko et al., 1999). Increases in K^+ efflux and intracellular K^+ depletion activated interleukin-1 β -converting enzyme in macrophages and monocytes (Devary et al., 1991; Derijard et al., 1994; Li et al., 2002), lowering intracellular K^+ concentration activated caspase-3-like proteases and apoptosis (Bortner et al., 1997). These studies suggest that UV-induced K^+ channel activation plays a critical role in eliciting excessive K^+ efflux or intracellular K^+ depletion. This is also consistent with studies that show neuronal apoptosis induced by serum deprivation, staurosporine, α -amyloid peptide or ceramide enhanced an outward K^+ current, which is prevented by a K^+ channel blocker and by increasing extracellular K^+ concentration (Jumblatt, 1997; Guo et al., 1998; Kaneko et al., 1999).

In our recent reports, exposure of corneal epithelial cells to UV-C irradiation ($42 \mu\text{J}/\text{cm}^2$) provokes activation of a plasma membrane K^+ channel. UV irradiation activates in rabbit corneal epithelial cells robust K^+ channel activity at both whole-cell and single channel levels measured with the nystatin-perforated whole-cell and cell-attached patch clamp configurations, respectively. First, the whole-cell current was recorded by depolarization of the membrane potential from a holding potential of -60 to $+60$ mV in 20 mV increments using nystatin-perforated patch clamping. Amplitudes of the whole-cell K^+ current were rapidly increased within 1 min after exposure of cells to UV irradiation (Fig. 5A). UV-evoked K^+ current is sensitive to 4-AP and is completely blocked in the presence of 1 mM 4-AP in the patch clamp chamber. The amplitude of the K^+ current is doubled within 1 min after exposure to UV light and reaches its maximal amplitude within 5 min. Normalized K^+ currents in the presence and absence of 4-AP were determined by the fractions of UV-induced current amplitudes and currents measured in control patches (I_{UV}/I_C), where I_{UV} and I_C represent currents were measured with and without UV irradiation, respectively (Fig. 5A). The effect of UV irradiation on single K^+ channel activity is further verified by the cell-attached single patch clamp and single K^+ channel activity (NPo) in corneal epithelial cells at a membrane potential of -60 mV is recorded in vivo. Exposure of cells to UV irradiation strongly evoked K^+ channel activity (Fig. 5B). Single channel activity was suppressed by application of 100 μM 4-AP in the patch pipette. UV-irradiation fails to activate K^+ channel activity in the presence of 4-AP. Time courses of UV-induced increase in K^+ channel activity are also studied at the single channel level. UV irradiation induces a rapid increase in K^+ channel activity with a time constant of 53 s and 100 μM 4-AP effectively blocks UV-induced K^+ channel activity. Statistical analysis of NPo is performed in 18 independent patches with and without adding 4-AP in the patch pipette. Upon UV irradiation, K^+ channel activity is increased from $17 \pm 3.6\%$ to $67 \pm 7.6\%$ within 1 min, which is fairly consistent to the result obtained from the whole-cell recording. These results indicate that an early effect of UV irradiation is direct stimulation of cell membrane K^+ channel activity in corneal epithelial cells.

3. UV irradiation-induced corneal epithelial cell apoptosis

3.1. UV irradiation-induced cell shrinkage

K^+ channels are known to play a major role in cell volume regulation (Grinstein and Foskett, 1990; Lang et al., 1990). This function has been proposed, at least in lymphocytes, to control mitogenesis indirectly (Gelfand et al., 1986; Krause et al., 1988; Deutsch, 1990). It has been found that when lymphocytes are exposed to a hypotonic medium, they swell and then come back towards their initial volume (Deutsch et al., 1982; Deutsch and Chen, 1993). This regulatory volume decrease (RVD) is mediated by increases in both K^+ and Cl^- channel activity. RVD in mitogen-stimulated cells could prevent an increase in size that may have resulted from an increase in nutrient uptake and from an increase of the membrane surface area. In this case, RVD mediated by K^+ channel activity would represent a protective response

allowing cells to progress normally through the cell cycle (Deutsch, 1990). A recent study shows that blockade of K^+ channel activity-induced cell swelling resulted in inhibition of neuroblastoma cell proliferation (Rouzaire-Dubois and Dubois, 1998). Now, it is clear that K^+ channels play an important role in volume regulation.

It has been shown that a hallmark of apoptosis is cell shrinkage. A significant increase in K^+ efflux is another event that occurs prior to apoptosis-induced cell shrinkage (Dallaporta et al., 1998). Recent evidence implies that the apoptosis-induced K^+ channel activation plays a critical role in eliciting excessive K^+ efflux or intracellular K^+ depletion (Yu et al., 1997). Blocking K^+ efflux by a K^+ channel blocker or by increasing extracellular K^+ inhibited shrinkage and apoptosis in human eosinophil (Beauvais et al., 1995a) human myeloid HL-60 cells (McCarthy et al., 1994), and mouse cortical neurons (Yu et al., 1999a). The K^+ channel blocker 4-AP can prevent cell shrinkage of human eosinophils undergoing apoptosis induced by cytokine withdrawal, and a combination of two K^+ channel blockers, TEA and 4-AP, inhibited IL-1 β release from lipopolysaccharide (LPS)-stimulated monocytes (Beauvais et al., 1995a,b; Walev et al., 1995). Neurons undergoing apoptosis exhibited an upregulation of outward K^+ currents by the K^+ channel opener cromakalim (Yu et al., 1997). In the present study, we demonstrate that UV irradiation-induced robust activation of these channels appears to be responsible for K^+ efflux and the consequent membrane hyperpolarization, thereby activating a particular intracellular signaling system(s) leading to corneal epithelial cell apoptosis. K^+ channel activity stimulation can indeed result in the loss of intracellular K^+ in addition to cell volume shrinkage (Barbiero et al., 1995; Hughes et al., 1997). Either one of these alternatives, K^+ efflux due to stimulation of the K^+ channel activity or rapid loss of K^+ leading to cell shrinkage, could be an early upstream event in the signaling cascade in corneal epithelial cells, leading to apoptosis. Alternatively, cell shrinkage that occurs as a result of a quick fall in intracellular K^+ concentration, may trigger apoptosis. Accordingly, suppression of K^+ channel activity may prevent rapid loss of intracellular K^+ ions resulting from UV-induced K^+ channel hyperactivity. This possibility is supported by recent findings that UV irradiation-induced JNK activation can be mimicked by hypertonic stress in Hela cells (Rosette and Karin, 1996). In addition, cytokine receptors can be activated by either UV irradiation or hypertonic shrinkage (Yu and Choi, 2000). It is speculated that cytokine receptor activation induced by hypertonic stress occurs as a consequence of cell shrinkage.

3.2. Theoretic model of UV-induced cell shrinking due to loss of $K^+_{[In]}$

According to the data collected from our patch clamp experiments in these cells, a theoretic model can be used to examine the hypothetical probability of UV-induced K^+ channel hyperactivity causing cell shrinkage. Our hypothesis is approachable under several assumptions: (1) considering the cell as a sphere, and do not change the shape over time; (2) considering the capacitance of the cell keeping constant, as $C = A \times D = 4\pi d$, (where A is the whole membrane area, C is the capacitance of the cell, D is dielectric constant, d is the thickness of the membrane), C keeps constant as far as the A keeps unchanged, which is a reasonable assumption; and (3) no physiological machineries that kick in as early as UV-induced K^+ channel activation, for instance, Na/K pump, cotransporters, exchangers, etc. Assumption number 3 is included in order to simplify the matter of facts that assume no other mechanisms involved, test the impacts of K^+ channel hyperactivation in response to UV irradiation on changes of intracellular K^+ concentration, and verify whether by K^+ channel hyperactivation alone it is sufficient to cause the speculative osmotic shock and volume change. From our data collected in whole-cell and single-channel patch clamp, a corneal epithelial cell has a whole-cell K^+ current of 167 ± 11 pA, with a measured membrane potential of 43.2 ± 2.5 mV at control level and the current rises to 680 ± 55 pA, while the membrane potential drops to -61.2 ± 1.0 mV after stimulation by UV irradiation. The average cell size is 19–21 μ m in diameter. Intracellular K^+ concentration is around 140 mM and extracellular K^+ concentration is about

2 mM. The average whole-cell capacitance is 21 pF. At the control level, the outward K^+ current is balanced by other mechanism(s), most likely Na/K pump and Na-K-2Cl cotransporter (NKCC). After UV irradiation, the K^+ current increases about 3–4 times, and there is a membrane potential drop of 18 mV. From the increase of the K^+ current, we can calculate the Q value (the net efflux of K^+ ion) by the following equation:

$$Q = [(680 - 167) \times 10^{-12} \text{ coulomb/s}] / 96,487 \text{ coulombs/mole} \\ = 5.3 \times 10^{-15} \text{ moles/s.} \quad (1)$$

The net charges needed for the membrane potential drop would be:

$$\Delta Q = C \times \Delta \Psi = 21 \times 10^{-12} \text{ F} \times (61.2 - 43.2) \times 10^{-3} \text{ V} \\ = 3.8 \times 10^{-13} \text{ coulombs} = 3.9 \times 10^{-18} \text{ moles.} \quad (2)$$

To comparing charges needed for the membrane potential drop with the net K^+ current, this number obtained from the Eq. (2) is negligible. We can consider there is a net flow of anion at the same speed to neutralize the cation movement. Therefore, there should be a net ion flow at the rate of 1.06×10^{-14} moles/s from the intracellular toward extracellular side. Now considering the impact of K^+ loss to intracellular K^+ concentration, let us convert the K^+ loss of 5.3×10^{-15} moles/s to intracellular K^+ concentration drops. As the diameter of the cell is 20 μm , the volume of the cell (V) is

$$V = 1/6 \pi d^3 = 1/6 \times 3.14 \times (2 \times 10^{-5})^3 \text{ m}^3 = 1.05 \times 10^{-12} \text{ L.} \quad (3)$$

Thus,

$$\Delta c_K = (5.3 \times 10^{-15} \text{ moles/s}) / (1.05 \times 10^{-12} \text{ L}) = 5.0 \text{ mM/s.} \quad (4)$$

As intracellular K^+ concentration is 140 mM, the net K^+ current deprives nearly 3.6% of the total intracellular K^+ stock per second. If osmotic pressure change is considered, the change may be 10 mOsm/s, which is more than 3% of the intracellular osmolarity. If the other mechanisms would not kick in at a timely fashion, the total intracellular K^+ could be depleted in 32 s. According to our data, this K^+ channel keeps opening for many minutes. When the net ion flow would be 1.06×10^{-14} moles/s as calculated above, the change of cell volume (dV) in a short time lapse (dt) can be estimated in normal isotonic conditions (300 mOsm) as the following:

$$dV/dt = (1.06 \times 10^{-14} \text{ mole/s}) / (0.3 \text{ Osm}) = 3.5 \times 10^{-14} \text{ L/s.} \quad (5)$$

Definitely, there are some other transport machineries that are activated to counterbalance this dramatic K^+ loss. However, at the first second or a few seconds, it is still a good assumption that the K^+ channel is the only machinery being stimulated. In conclusion, the theoretical calculation supports our hypothesis. It shows that UV-induced hyperactivity observed in PRCE cells is potent enough to cause dramatic osmotic pressure changes and lead to cell shrinkage. The computation demonstrates a good hypothetical probability and gives us a direction for further investigations.

3.3. Effect of losing intracellular $[K^+]$ on cell viability

Valinomycin, a K^+ ionophore, forms membrane pores that allow K^+ ions passing through following electrical and chemical gradients across the cell membrane. It has been shown that valinomycin induces apoptosis in various cell types including lymphocytes, tumor cells and

mouse neocortical neurons. In a previous study, corneal epithelial cells are exposed to valinomycin (100 nM) for 24 and 48 h to trigger apoptosis measured by both DNA fragmentation and nuclear EB/AO staining (Lu et al., 2003). Following exposure to valinomycin concentration gradient, valinomycin induces a clear pattern of DNA laddering and increases DNA condensations at both 24 and 48 h time points, indicating internucleosomal DNA cleavage in rabbit corneal epithelial cells (Lu et al., 2003). The ability of an increase in K^+ efflux to elicit cell apoptosis supports the notion that intracellular declines in K^+ due to UV-induced K^+ channel hyperactivity may be a cause of cell death.

3.4. UV irradiation-induced cellular damage

The physiological balance between the ability of the corneal epithelium to proliferate and programmed cell death (apoptosis) contributes to the maintenance of corneal deturgescence, transparency and normal vision (Pepose and Ubels, 1992; Wilson et al., 1992a,b; Mohan et al., 1997; Wilson, 1997). There is definitive evidence that UV irradiation at wavelengths in the range from 280 to 310 nm induces apoptosis in the corneal epithelium based on terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL)-positive staining in the intact rabbit and rat corneas (Tronnier, 1980; Podskochy et al., 2000; Wang et al., 2003). Recently, it has been shown that exposure to UV is the leading cause of post-PRK corneal epithelial detachment and death (Nagy et al., 1995, 1997; Wilson, 1997; Johnson et al., 1998). There is recent biophysical evidence showing that UV light penetrates and is differentially absorbed by ocular tissues. Their absorption pattern in the antero-posterior direction follows a wavelength-dependent decade (Ringvold and Davanger, 1985; Ringvold, 1997, 1998). The corneal epithelium absorbs UV wavelengths shorter than 310 nm (UV-B between 290 and 320 nm and UV-C between 200 and 290 nm) thereby acting as a filter and protecting the lens and retina from UV-induced damage (Fig. 6). In doing so the corneal epithelium and lens protect the retina from UV irradiation-induced damage (Ringvold, 1998; Taylor et al., 1998; Podskochy et al., 2000). In addition, repeated subthreshold or single threshold UV-exposures lead to inhibition of mitosis, nuclear fragmentation and loss of the whole corneal epithelial layer. Disturbances in epithelial structure or function can lead to infection, development of corneal opacity and loss of vision (Wickert et al., 1999). UV irradiation-induced programmed cell death (apoptosis) in the cornea directly decreases viabilities of corneal epithelial cells, and especially affects those actively dividing progenitor cells. UV damage in the cornea can result in delayed or retarded responses of wound repair, and make corneal epithelial cells become more susceptible to the injury caused by bacterial and viral infections. Stress-induced stimulation to the cornea, including cytokines, osmotic pressure and other chemical and physical stimulation, directly affect wound healing of corneal epithelial cells. UV irradiation is one of such stimuli causing stress responses of corneal epithelial cells. In the normal corneal epithelium and endothelium, it has been suggested that aromatic amino acids such as tryptophan, are in part responsible for absorbing UV. In addition, ascorbate may effectively absorb UV in the cornea and lens epithelium (Ringvold, 1997). However, when the system is overwhelmed by over dose of UV exposure, UV irradiation will induce apoptotic damage of these cells.

3.5. Kv channel mediating UV-induced apoptosis

Various investigations have shown that K^+ channel activity can be affected by apoptosis inducers, including ROS, Fas ligand and TNF, and anticancer drugs (Soliven et al., 1991; Dubois and Rouzaire-Dubois, 1993; Bright et al., 1994; Duprat et al., 1995; Szabo et al., 1996). There is growing evidence showing that K^+ channel activities are probably involved in programmed cell death in many cell types (Schulz et al., 1996; Eldadah et al., 1997; Yu et al., 1997; Wang et al., 1999b). The K^+ channel blocker 4-AP inhibits the shrinkage of human eosinophils undergoing apoptosis induced by cytokine withdrawal (Beauvais et al., 1995b), and a combination of two K^+ channel blockers, TEA and 4-AP, inhibited IL-1 β release from

LPS-stimulated monocytes (Walev et al., 1995). Neurons undergoing apoptosis exhibited an upregulation of outward K^+ currents. This enhancement of outward K^+ current, induced by serum deprivation and staurosporine, can be prevented by the K^+ channel blocker TEA and by increasing the extracellular K^+ concentration. It has also been observed that the K^+ channel opener cromakalim induces neuronal apoptosis (Yu et al., 1997). Thus, it appears that the activation of K^+ channels is responsible for K^+ efflux and the consequent membrane hyperpolarization and decrease in cell volume thereby activating a particular signaling system leading to activation of caspase cascades and apoptosis.

Increases in K^+ efflux and intracellular K depletion activate interleukin- 1β -converting enzyme in macrophages and monocytes (Devary et al., 1991; Derijard et al., 1994; Li et al., 2002). Lowering intracellular K^+ concentration activates caspase-3-like proteases and apoptosis (Ashkenazi and Dixit, 1998; Wang et al., 1999a). Furthermore, neuronal apoptosis induced by serum deprivation, staurosporine, α -amyloid peptide or ceramide enhances an outward K^+ current, which was prevented by a K^+ channel blocker and by increasing extracellular K^+ concentration (Yu et al., 1997, 1998, 1999a, Yu et al., b). In addition, UV irradiation induces apoptosis in human myeloblastic leukemia ML-1 cells through activation of a membrane K^+ channel. Blockade of UV-induced K^+ channel activity with 4-AP completely prevented UV-induced apoptosis in ML-1 cells (Wang et al., 1999a).

A recent report indicates that UV irradiation results in apoptosis in corneal epithelial cells through activation of a membrane K_v channel (Wang et al., 2003). In fact, UV irradiation-induced hyperactivation of cell membrane K^+ channels is an important early component in the signaling process to mediate cell apoptosis (Wang et al., 1999a, 2003). The effect of blocking K^+ channel activity with the K^+ channel inhibitor, 4-AP, on UV irradiation-induced apoptosis is determined by comparing cell viability in UV irradiated cells and anti-tumor drug etoposide-treated cells. Exposure of corneal epithelial cells to both UV irradiation and etoposide results in decreased cell viability determined by orange-stained nuclei indicating nuclear death of the cells. Suppression of K^+ channel activity with 4-AP protected these cells from UV irradiation-induced nuclear death, whereas it is ineffective in preventing etoposide-induced nuclear death. In the presence of 1 mM 4-AP, rabbit and human corneal epithelial cell viability is well protected from UV-irradiation (>98% for control, >96% for 4-AP-treated, 36–40% for UV-induced and >91% for UV plus 4-AP) (Fig. 7A). Other groups of corneal epithelial cells are treated with etoposide (an inhibitor of topoisomerase II), indicating that 4-AP has no protective effect on etoposide-induced cell death. With etoposide alone, viability decreased to 45–50%. This decline is indistinguishable from the effect of etoposide measured in the presence of 4-AP. The effects of blocking channel activity on UV- and etoposide-induced cell apoptosis are further confirmed by using DNA fragmentation assays. Suppression of K^+ channel activity with 4-AP completely prevents UV-induced DNA fragmentation in corneal epithelial cells, but does not prevent etoposide-induced DNA fragmentation (Lu et al., 2003). These results reveal that UV irradiation elicits K^+ channel hyperactivity, which, in turn, mediates apoptosis. The ability of etoposide to induce apoptosis despite the presence of 4-AP is because its effect bypasses the membrane events, which is consistent to the known inhibitory effect of topoisomerase II activity at the level of the nucleus.

In addition, various techniques, including DNA fragmentation, nuclear staining, caspase 3 activation and TUNEL assay, are used to detect UV-induced apoptosis in the corneal epithelium (Lu et al., 2003; Wang et al., 2003). For example, H-E staining revealed a normal corneal epithelial histological structure in cultured rat corneas (Fig. 7B, panel a). There was no positive TUNEL staining detected in the basal layer of the corneal epithelium and a few positive staining limited to the superficial epithelial cells in the control cornea (Fig. 7B, panel b). However, UV irradiation-induced nuclear condensations in all layers. Especially, there was a mass of positive staining found in the basal layer of the corneal epithelium. In addition, UV irradiation markedly

reduced the thickness of the corneal epithelium (Fig. 7B, panel c). In contrast, there was no positive staining in any epithelial layers of the cornea treated with 1 mM 4-AP, indicating that suppressing K^+ channels with 4-AP prevented the corneal epithelium from UV irradiation-induced apoptosis (Fig. 7B, panel d). These results firmly state that UV irradiation-induced apoptosis of the corneal epithelium requires activation of membrane K^+ channels. In parallel to the involvement of K^+ channels in UV irradiation-induced corneal epithelial cell apoptosis, suppression of Kv channel activity can also prevent UV irradiation-induced apoptosis in human myeloblastic ML-1 cells (Wang et al., 2003).

4. UV-induced activation of the JNK signaling pathway

4.1. UV-induced K^+ channel and JNK activation

Interestingly, UV irradiation-induced cellular responses share common signaling mechanisms in cytokine- and other stress-induced pathways that relate to the programmed cell death. Thus, it is necessary to understand UV irradiation-induced cellular responses in order to further study wound healing of corneal epithelial cells. Previous studies indicate that exposure of cells to UV causes increases in membrane EGF, TNF and CD95/FasL receptor phosphorylation and clustering (Rosette and Karin, 1996; Bender et al., 1997; Schwarz, 1998). In addition, these cytokine receptors can also be activated by hypertonic stress (O'Neill, 1995). Several signaling components have been identified in response to UV-induced cytokine receptor activation including *Ras-Raf*, *Src*, and *caspases*. Recent studies also indicate that stimulation of K^+ channel activity causes the quick loss of intracellular K^+ , which results in activation of caspase cascades (Walev et al., 1995; Frisch et al., 1996; Bortner et al., 1997). The effect of fast loss of intracellular K^+ on caspase activation suggests that there is a crosstalk occurring between activation of the K^+ channel and intracellular signaling pathways. There is accumulating evidence that support the existence of such a crosstalk, including: (1) voltage-gated K^+ channel activity involved in cell proliferation (Lu et al., 1993; Xu et al., 1996); (2) Kv channel activity regulated by growth factors (Xu et al., 1999; Roderick et al., 2003); and (3) Kv channel activity associated with *Src* tyrosine kinase (Holmes et al., 1996; Nitabach et al., 2001). More importantly, portions of the MAP kinase signaling pathways, including JNK/SAPK and p38 signaling pathways, mediate UV irradiation-induced corneal epithelial cell apoptosis (Adler et al., 1996; Price et al., 1996; Merienne et al., 2000; Boderio et al., 2003; Lu et al., 2003). Activation of ICE (caspase 1) can affect upstream events in the JNK pathway at the JNK level (Kong et al., 1998; Lei et al., 1998). We found that UV irradiation-induced activations of ICE and JNK-1 occur subsequent to the stimulation of K^+ channel activity and the loss of intracellular K^+ in corneal epithelial cells (Lu et al., 2003). Activation of the JNK signaling pathway by UV irradiation finally results in apoptosis. After UV irradiation, JNK-1 activities are markedly increased following a time course up to 60 min compared to control groups in corneal epithelial cells (Fig. 8A). UV irradiation-induced JNK-1 activation is initially observed within 5–15 min after the end of the exposure period. This observation is supported by other findings that the UV irradiation-induced JNK activation can be mimicked by heat shock and hypertonic stress in Hela cells (Adler et al., 1995; Rosette and Karin, 1996).

The effect of K^+ efflux on inhibition of JNK activation is determined by studying the association between changes in K^+ channel activity and JNK activity in corneal epithelial cells. Suppression of UV-induced K^+ channel activation with specific channel blockers, including 4-AP, Ba^{2+} and TEA (tetra-ethylammonium), sufficiently prevented UV irradiation-induced activation of JNK (Fig. 8B). The inhibitory effect of these blockers on JNK signaling pathway is rather specific because there is a good correlation for the blockers to inhibit UV irradiation-induced JNK activation and Kv channel activity. The dose-response curve demonstrates that 4-AP effectively inhibits JNK activity with an IC_{50} of $\sim 170 \mu M$ (Wang et al., 1999a; Lu et al., 2003). This IC_{50} value is very close to the IC_{50} value that is required for 4-AP to block the K^+ current in patch clamp studies. IC_{50} values for 4-AP to inhibit Kv3.4 channels are from 80

to 120 μM dependent upon the membrane potentials (Fig. 8C) (Lu et al., 1993; Wang L et al., 2004). In general, blockade of K^+ channel activity using 4-AP (1 mM) significantly prevents the UV irradiation-induced JNK-1 activation in corneal epithelial cells. These results indicate that UV irradiation stimulates JNK-1 activities in corneal epithelial cells and this activation is associated with UV irradiation-evoked increases in cell membrane K^+ channel activity. These results also indicate that there is a crosstalk between UV irradiation-induced activation of the Kv channel and the JNK signaling pathway.

4.2. Dependence of JNK activation on K^+ efflux

Activation of the Kv channel induces K^+ effluxes following the electrical-chemical gradient of K^+ ions across the membrane potential of corneal epithelial cells. As described above, UV irradiation-induced hyperactivity of Kv channel causes sudden increases in K^+ efflux resulting in activation of JNK. The effect of K^+ efflux on intracellular signaling pathways can be mimicked by applying valinomycin, a K^+ ionophore, to cells. Exposure of corneal epithelial cells to valinomycin markedly increases JNK activity in a dose dependent manner (Lu et al., 2003). Inhibition of K^+ channel activity using 4-AP does not affect valinomycin-induced JNK activation in these cells. Thus, valinomycin treatment-stimulated JNK activity in a dose-dependent manner provides further supports that a rapid loss of intracellular K^+ ions due to K^+ channel activation constitutes an early event in the UV irradiation-activated JNK signaling pathway resulting in cell apoptosis.

4.3. UV irradiation-induced K^+ channel and SEK activation

SEK is a MAP kinase immediately upstream of JNK in the JNK signaling pathway. Activation of SEK results in the phosphorylation and activation of JNK (Sanchez et al., 1994; Auer et al., 1998; Wang et al., 1999a). The effect of UV irradiation-induced hyperactivity of K^+ channel on SEK activation is observed by measuring the phosphorylation status of SEK. SEK-1 protein is strongly phosphorylated in response to UV irradiation following a time course of 60 min. Suppression of K^+ channel activity with 4-AP effectively prevents UV irradiation-induced SEK-1 phosphorylation. This result indicates that UV irradiation-induced increases in K^+ channel activity also affect early events upstream from SEK in the JNK signaling pathway (Lu et al., 2003; Wang et al., 2005).

4.4. Independence of JNK activation on Ca^{2+} influx

Since changes in K^+ channel activity can alter the membrane potential and cause increases in Ca^{2+} influx, a rise in Ca^{2+} influx may play a role in activating intracellular signaling pathways that mediate UV irradiation-induced corneal epithelial cell apoptosis. To determine whether such an effect is dependent on a rise in Ca^{2+} influx, the effect of UV irradiation on JNK activation is determined in a nominally Ca^{2+} -free condition by adding 5 mM EGTA in the extracellular medium. It is interesting that UV irradiation has the same effect on JNK activation in the Ca^{2+} -free medium in both corneal epithelial and myeloblastic ML-1 cells (Wang et al., 1999a; Lu et al., 2003). However, suppression of K^+ channel activity with 4-AP prevents the UV irradiation-induced JNK activation under this condition. Although this experiment does not determine the effect of Ca^{2+} release from intracellular compartments, it suggests that Ca^{2+} influx does not apparently play a role in the UV irradiation-mediated JNK cascade activation in these cells.

4.5. Independence of hypertonic stress-induced JNK activation on K^+ channel activity

Hyperosmotic stress induces cell apoptosis by activating JNK and p38 signaling pathways in various cell types (Qin et al., 1997; Bilney and Murray, 1998; Malek et al., 1998; Hoover et al., 2000; Lu et al., 2000; Morrison et al., 2003; Reinehr et al., 2003). In corneal epithelial cells, the effect of hyperosmotic stress on apoptosis is characterized by measuring cell viability and

activations of JNK and p38 signaling pathways. Cell viability is determined based on nuclear EB/AO staining. Corneal epithelial cell viability is significantly decreased following an increase in sorbitol concentration that created a hyperosmotic stress (Lu et al., 2003). To further support the specific role of K⁺ channel activity in the UV irradiation-induced activation of JNK cascade, the effect of 4-AP on JNK-1 activation is determined by treating corneal epithelial cells with a hypertonic stress of 600 mmol/L. This challenge markedly evokes JNK-1 activity, but 4-AP had no inhibitory effect on this activation, indicating that sorbitol-induced JNK activation is not dependent on activation of a 4-AP-sensitive K⁺ channel activity in the membrane in these cells. In addition, sorbitol induces a strong activation of p38. Similarly, inhibition of K⁺ channel activity by 4-AP has no effect on sorbitol-induced p38 activation. These results further support the notion that the UV irradiation-induced JNK activation is likely mediated by activation of a 4-AP sensitive K⁺ channels in the membrane (Lu et al., 2003). In addition, these results indicate that the UV irradiation-induced activation of JNK does not share the same signaling pathways induced by hypertonic stress in corneal epithelial cells.

4.6. Dependence of UV-induced ERK or p38 activation on K⁺ channel activation

In many different cell types, UV irradiation can induce increases in the activity of two other limbs of the MAP kinase signaling cascade: ERK and p38 (Radler-Pohl et al., 1993; Li et al., 2002; Wang et al., 2003). Exposure of corneal epithelial cells to UV irradiation also induces activation of the ERK limb. In corneal epithelial cells, UV irradiation induces a weaker activation of the ERK than the JNK/SAPK limb, which is consistent with previous studies in other cell types (Lu et al., 1996; Karin et al., 1997; Li et al., 2002). Time-dependent activation of Erk-2 by UV irradiation is measured to determine the characteristic of UV irradiation-induced effects on the ERK signaling pathway. Following UV irradiation, Erk-2 activity increases 5 min later and its activation reaches a plateau after 15 min. These effects are significantly inhibited by suppression of K⁺ channel activity using 4-AP (1 mM), suggesting that K⁺ channel activity is required for UV irradiation-induced activation of the ERK cascade. The functional significance of UV irradiation moderately and stably activating the ERK limb may be explained by a previous study demonstrating that the ERK limb activation by UV irradiation promotes cell survival in A431 cells (Kitagawa et al., 2002). Relative to JNK/SAPK activation, there is also a much weaker activation of p38 in corneal epithelial cells in response to UV irradiation. UV irradiation induces a time dependent, but weak activation of the p38 limb (Lu et al., 2003). Suppression of K⁺ channel activity by 4-AP does not block the effect of UV irradiation on p38 activation (Lu et al., 2003; Wang et al., 2003). In summary, suppression of K⁺ channel with specific K⁺ channel blocker, 4-AP, markedly inhibits UV irradiation-induced JNK and Erk activations, indicating that UV irradiation-induced K⁺ channel activation plays an important role in eliciting activation of downstream kinase cascades. However, it is not known why UV irradiation of corneal epithelial cells elicited a smaller increase in p38 activity than in other cell types.

5. The UV-induced K⁺ channel activity involving p53 activation

5.1. Role of p53 in UV irradiation-induced DNA damage

It is known that p53 is multi-functional in various cell types. Activation of p53 in response to a variety of cellular stresses including UV irradiation is a crucial component of cellular mechanism. Although the mechanisms underlying p53-dependent apoptotic responses remain incompletely characterized, p53 is known to be involved in both the extrinsic and the intrinsic pathways of apoptosis by initiating apoptosis through mitochondrial depolarization and sensitizing cells to inducers of apoptosis (Wang et al., 1998, 1999a,b; Haupt et al., 2003; Tang et al., 2003). The phosphorylation status of p53 determines the stability of the protein and controls cell cycle progression, which serves as a master-switch for promoting apoptosis (Ginsberg et al., 1991; Ashcroft and Vousden, 1999; Colman et al., 2000; Asher et al.,

2001;Sogame et al., 2003). Alterations of p53 protein, such as missense mutations and loss of its expression caused by nonsense or frame-shift mutations, can result in carcinogenesis (Hussain and Harris, 1998;Medina et al., 2002;Shirai et al., 2002;Nishikawa et al., 2003;Hofseth et al., 2004).

In previous studies, p53 has been described as a major molecule executing UV irradiation-induced DNA damage (Abrahams et al., 1995;Smirnova et al., 2001). The signaling pathways underlying the cellular response to DNA damage (genotoxic stress) consist of sensors, signal transducers, and effectors (Zhou and Elledge, 2000). Although the identities of the damage sensors remain unknown, the molecular entities responsible for transducing the damage signals to specific effectors are relatively well characterized. For example, one of its roles is to act as a tumor suppressor protein that senses DNA damage and acts as a guardian of genome stability (Chernova et al., 1995;Blaise et al., 2001;Gentiletti et al., 2002). In some cases, p53 becomes a molecular signature based on the type of cancer (Hussain et al., 2000). The multiple functions of p53 include: (1) to regulate cell-cycle progression; (2) to survey cellular stress; and (3) to induce apoptosis in extreme cases. The cellular event involving active p53 is to stimulate the apoptotic infrastructure by increasing the expression of apoptotic protease-activating factor 1 (APAF-1), a crucial component of the apoptosome (Kinzler and Vogelstein, 1997;Levine, 1997). Functional proteins downstream from the p53 interactive signaling pathways, such as ataxia telangiectasia mutated (ATM) and homolog of ATM (ATR), are central to the DNA damage response (Zhou and Elledge, 2000;Goodarzi et al., 2003;Dodson et al., 2004;Irrarazabal et al., 2004;Kastan, 2004;Yang et al., 2004). Further downstream targets (substrates) of ATM/ATR include the checkpoint protein kinases Chk1 and Chk2 (Ma et al., 1998;Matsuoka et al., 1998;Sanchez et al., 1999;Tominaga et al., 1999). Chk1 and Chk2 are thought to mediate different types of DNA damage to the specific cellular responses.

5.2. UV irradiation-induced activation of p53 in corneal epithelial cells

There is very little information up to date about the expression and function of p53 in corneal epithelial cells. However, a recent study from our lab has provided new data to demonstrate that there are high expression levels of p53 protein in corneal epithelial cells. UV irradiation in these cells induces two stages (earlier and later) of cellular events that subsequently involve activation of p53 (Wang et al., 2005). Expression levels of p53 protein in hypo-phosphorylated states are highly expressed in corneal epithelial cells. At the cellular level, the amount of p53 proteins in phosphorylation status are not altered by passages in the cultured condition in both types of cells. Phospho-p53 protein at different serine sites is detectable in corneal epithelial cells using antibodies specific to phospho-serine residues. UV irradiation clearly induces an increase in phosphorylation levels of p53 following a time course at Ser15 and Ser20, while there is no change in phosphorylation of p53ser46. This result indicates that phosphorylation of p53 in response to UV irradiation in RCE cells is site-specific (Fig. 9A). UV irradiation-induced p53ser15 phosphorylation (within 5–15 min) occurs earlier than the site of p53ser20 (in 30 min). These data are consistent to the results obtained from Western analysis of this protein (Wang et al., 2005). Immunostaining experiments reveal that UV irradiation increases phosphorylation of p53ser15 in the nuclei in 15 min, but total p53 protein levels are not changed in control cells (Fig. 9B). These results conclude that there are high levels of p53 expressed in corneal epithelial cells and UV irradiation activates p53 by phosphorylating both ser15 and ser20 sites.

It has been shown that phosphorylation of p53 in response to stress stimulation may be partially resulted from activation of the JNK signaling pathway. To investigate the correlation of JNK activation and p53 phosphorylation, the interaction between JNK and p53 in corneal epithelial cells is studied by employing several approaches. First, time courses of UV irradiation-induced increase in phosphorylation levels of JNK and p53 proteins demonstrate that the UV

irradiation-induced JNK phosphorylation occurs earlier than phosphorylation of p53ser15, suggesting that phosphorylation of p53ser15 is a further downstream event in the pathway. Second, JNK1 and p53 proteins can be pulled down in UV irradiation-induced rabbit corneal epithelial cells in immunoprecipitation experiments by anti-JNK1 antibodies. Finally, JNK1 purified by immunoprecipitation from UV irradiated corneal epithelial cells is able to catalyze phosphorylation of GST-p53 at ser15. These results are consistent with previous studies indicating that p53 is a substrate of JNK and that there is an interaction between JNK and p53 in other cell types (Fuchs et al., 1998a,b; Pluquet et al., 2003).

5.3. Effect of suppressing Kv channels on UV-induced p53 phosphorylation

As it has been mentioned above, suppression of Kv channels prevents UV irradiation-induced apoptosis in corneal epithelial cells because the UV irradiation-elicited Kv channel hyperactivity results in activation of JNK cascades (Lu et al., 2003). In corneal epithelial cells, inhibition of Kv channels with various Kv channel blockers prevents UV irradiation-induced JNK1 activation and effectively suppresses phosphorylation levels of p53 (Fig. 10). More specifically, the UV irradiation-induced phosphorylation of p53ser15 is markedly suppressed by 4-AP in a similar dose range that inhibits the UV irradiation-induced JNK phosphorylation. The inhibitory effect of different Kv channel inhibitors on p53ser15 phosphorylation is sustained up to 8 h. Among these Kv channel blockers, 4-AP (1 mM) effectively inhibited about 50% of p53ser15 phosphorylation. Other blockers in the group, including Ba²⁺ (5 mM), α -DTX (at 200 nM) and BDS-I (at 400 nM), partially inhibit the effect of UV irradiation on p53ser15 phosphorylation. The inhibitory effectiveness of these blockers is consistent with electrophysiological results obtained from patch clamp studies in corneal epithelial cells. Phosphorylation of p53 involving Kv channel activity and JNK cascades reveals that p53 is indeed a downstream component in the JNK signaling pathway in corneal epithelial cells.

5.4. Two pathways involving UV-induced phosphorylation of p53ser15

There is an other interesting observation showing that suppression of Kv channels with 4-AP (1 mM) can completely prevent UV irradiation-induced JNK activation, but only partially prevent UV irradiation-induced phosphorylation of p53ser15 (Wang et al., 2005). To understand why suppression of Kv channels only partially inhibits UV irradiation-induced p53ser15 phosphorylation in corneal epithelial cells, further investigation is conducted by using different UV stimulation protocols. Instead of a standard protocol using a fixed UV exposure for 3 min (42 μ J/cm²), an alternative protocol is used to treat cells with extensive UV irradiation by extending exposure time up to 30 min. Suppression of Kv channels with 4-AP in corneal epithelial cells partially prevented phosphorylation of p53ser15 within 15 min of UV exposure period. However, blockade of Kv channel failed to prevent phosphorylation of p53ser15 after 15 min of continuous exposure of these cells to UV irradiation (Fig. 11A). Caffeine can specifically inhibit DNA damage-induced phosphorylation of p53ser15 (Costanzo et al., 2003; Ito et al., 2003). In continuous UV exposed corneal epithelial cells, application of caffeine blocks both early and late stages of p53ser15 phosphorylation (Fig. 11B). These results indicate in corneal epithelial cells that there is an effect involving the UV irradiation-induced DNA damage distinguishable from the effect of UV irradiation-induced activation of the membrane Kv channel-linked signaling pathway. Different effectiveness of 4-AP and caffeine in suppression of UV irradiation-evoked phosphorylations of p53ser15 supports the dual pathway notion. One of the pathways is the membrane Kv channel activity-mediated activation of JNK cascades. Suppression of this pathway with Kv channel blocker 4-AP can prevent UV irradiation-induced phosphorylation of p53ser15 in the early stage. The other pathway is DNA damage-induced signaling pathway. It has been suggested that the sensors in response to DNA damage resulting in p53ser15 phosphorylation involve ATM and ATR (Xie et al., 2001; Ye et al., 2001). Caffeine is able to block DNA damage-induced ATM and ATR responses and phosphorylation of p53ser15 can be specifically blocked by caffeine (Costanzo et al., 2003; Ito

et al., 2003). In corneal epithelial cells, suppression of DNA damage-induced signaling pathway with caffeine can effectively prevent UV irradiation-induced phosphorylation of p53ser15. Thus, the conclusion is that suppression of Kv channel activity inhibits UV irradiation-induced p53 phosphorylation occurring in the early stage. However, UV irradiation-induced DNA damage increases p53 phosphorylation levels in both earlier and later stages.

6. Future developments

In conclusion, the stress- and cytokine-stimulated activation of Kv channels mediates the process of corneal epithelial wound healing by elucidating how UV irradiation stress induces damage. A summarized scheme in Fig. 12 presents future study directions that are fully supported by our published data and preliminary studies. The UV irradiation-induced apoptotic signal transduction in corneal epithelial cells is initiated by hyperactivation of Kv channels in the cell membrane resulting in a fast loss of intracellular K^+ ions and shrinkage of cell volume. Subsequently, alterations of cell volume activate scaffold protein kinases, such as FAK, MLK1 and MEKK1, leading to cytoskeleton reorganizations and activation of JNK cascades. In addition, we found that the UV-induced JNK activation activates p53 by increasing p53 phosphorylation levels at various sites to trigger apoptosis (Wang et al., 2005). In fact, our data demonstrates that UV-induced activation of JNK cascades is essential for UV-induced corneal epithelial apoptosis (Lu et al., 2003). Apparently, cell volume changes cause a mechanical stimulation in cells to activate membrane-spanning proteins, such as integrins at the leading edge of cells, which is also the region where integrins first engage their ligands, resulting in activation of scaffold proteins FAK and paxillin, as well as *Src*. In fact, the phosphorylation of FAK and paxillin can be elicited in cultured cells by cyclical mechanical strain (Yano et al., 1996; Smith et al., 1997, 1998). FAK has been proposed to couple 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 JNK cascades (Dolfi et al., 1998; Schlaepfer and Hunter, 1998; Zhao et al., 1998; King et al., 1999; Almeida et al., 2000). On the other hand, scaffold protein MEKK1, an upstream mitogen activated protein kinase K (MAPKK) kinase of JNK cascades, is also associated with cytoskeleton reorganization and activated in response to cell volume changes (Kwan et al., 2001; Cross and Templeton, 2004; Lieber et al., 2004). Recent studies found that FAK physiologically interacts with MEKK1 identified by immunoprecipitation and co-localization in response to EGF stimulation (Yujiri et al., 2003). Thus, it is very likely in the corneal epithelium that FAK coordinates signals from inputs of UV-induced volume shrinkage to the JNK signaling pathway. In our pilot studies, we found in corneal epithelial cells that UV irradiation induces phosphorylation of several scaffold proteins and kinases that are related to apoptosis, including FAK, MEKK1, MLK1, SEK, and JNK. In fact, both MEKK1 and MLK1 are important early components in the JNK signaling pathway.

Blockage of the UV-induced K^+ channel hyperactivation effectively prevents UV-induced cell shrinkage and phosphorylation of these signaling components in the JNK signaling pathway, which provides a strong foundation for our future studies. However, the signaling mechanisms that link UV irradiation-induced K^+ channel hyperactivation to JNK activation resulting in apoptosis, are still largely unknown. We believe that there are other signaling components in the pathway that play important roles in the linkage of the UV irradiation-induced K^+ channel hyperactivity and activation of JNK cascades in these cells. It is very likely that the effect of UV-induced stress response and apoptosis depends on the magnitude of the loss of intracellular K^+ ions, which in turn causes the shrinkage of cell volume. We now focus new and exciting studies to determine how UV irradiation-induced K^+ channel hyperactivity initiates rapid cell shrinkage resulting in the activation of scaffolding protein kinases and cytoskeleton reorganizations. Accordingly, we hypothesize that activation of JNK cascades and corneal epithelial apoptosis by UV irradiation requires shrinking cell volume and activating

cytoskeleton-related protein kinases upstream of the JNK signaling pathway. This prediction is based on theoretical calculations presented above and pertinent new data collected from our lab. We found that the effectiveness of UV-induced activation of JNK cascades and corneal epithelial cell apoptosis is dependent on the ability of UV irradiation to increase K⁺ channel activity, to decrease cell volume and to activate scaffolding kinases. We will undertake a new direction to address the following questions: (1) how K⁺ channel hyperactivity is stimulated by UV irradiation; (2) whether UV-induced cell shrinkage results in activation of scaffolding tyrosine kinase, FAK (focal adhesion kinase); and (3) what scaffolding tyrosine kinases activated by UV irradiation interact with upstream components in the JNK signaling pathway to trigger JNK cascades. Such studies will provide new insights into the mechanisms that underline corneal epithelial apoptosis resulted from stimulation of environmental stress and from release of cytokines in corneal injury.

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Abbreviations

4-AP	4-aminopyridine
AO	acridine orange
ATM	ataxia telangiectasia mutated
ATR	homolog of ATM
BDS-I	blood depressing substance-I
Chk	checkpoint protein
DTX	dendrotoxin
EB	ethidium bromide
EGF	epidermal growth factor
ERK	extracellular-regulated kinase
ET-1	

	endothelin-1
FBS	fetal bovine serum
GM-CSF	granulocyte/macrophage-colony stimulating factor
IFN-γ	interferon - γ
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
NGF	nerve growth factor
NKCC	Na-K-2Cl cotransporter
ROS	reactive oxygen species
RB	retinoblastoma protein
RVD	regulatory volume decrease
TEA	tetra-ethylamonium
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling
UV	ultraviolet

Fig 1. Distribution of Kv3.4 channels in corneal epithelium. (A) Expression of Kv3.4 channel in the corneal epithelium. Kv3.4 channel immunoreactivity was localized by confocal images in the membrane of cultured rabbit corneal epithelial cells (a) and rat corneal epithelium (b). (B) Detection of Kv channel protein expressions in rabbit corneal epithelial cells by Western analysis using a panel of various antibodies.

Fig 2.

Effects of specific Kv channel blockers on K⁺ current in rabbit corneal epithelial cells. (A) Time course of K⁺ current blocked by various concentrations of α -DTX and 4-AP. K⁺ currents were normalized as a fraction of I_{DTX}/I_C ; where I_{DTX} and I_C represent the peak K⁺ current measured before and after addition of α -DTX, respectively. (B) I-V relationship measured before and after addition of 400 nM BDS-I. Whole-cell currents were recorded by depolarization of the membrane potential from a holding potential of -60 to +60 mV for 2 s duration in 20 mV increments.

Fig 3. Growth factor-stimulated single K^+ channel activity in rabbit corneal epithelial cells. (A) Current-voltage relationship and K^+ selectivity of the channel. Cell-attached single channel recording was performed in symmetrical 145 mM KCl/145 mM KCl (●) or 145 NaCl/mM KCl (●) solution and the membrane potential was depolarized from a holding potential of -80 to $+80$ mV. (B) Statistic analysis of K^+ channel activities recorded from cell-attached patches stimulated by FBS, EGF, ET, and combination of EGF and ET for 10–20 min.

Fig 4. Effect of TNF- α on increases in K⁺ channel activity in human corneal epithelial cells. (A) Whole-cell current recorded from TNF- α -stimulated cells. Nystatin-perforated whole-cell patch clamp was performed to record K⁺ currents in response to TNF- α stimulation. I-V curves of K⁺ currents were obtained in cells 18 h after serum starvation and TNF- α stimulation. (B) Single K⁺ channel activity recorded in TNF- α -stimulated cells. Single channel activity was recorded in the absence and presence of 20 ng/ml TNF- α by the cell-attached patch clamp. The membrane potential was held at -60 mV and 4-AP (1 mM) or BDS-I (400 nM) was used to suppress TNF- α -induced K⁺ channel activity. (C) Effect of suppressing K⁺ channel activity on TNF- α -induced alterations of cell viability. Cells were treated with different dosages of 4-

AP 30 min prior to TNF- α stimulation (20 ng/ml). Cell apoptosis was determined by detecting chromatin condensation/fragmentation with EB/AO method at 36 h after TNF- α stimulation. Symbols “*” represent significant difference ($p<0.05$).

Fig 5.

Hyperactivation of 4-AP-sensitive K^+ current by UV irradiation. (A) Whole-cell currents were activated by exposure of rabbit corneal epithelial cells to UV light in the absence and presence of 4-AP. The membrane potential was depolarized from a holding potential of -60 to +60 mV in 20 mV increments. (B) Single K^+ channel activity activated by UV irradiation blocked by 4-AP. Cell-attached patch clamp experiments were performed in symmetrical 140 mM K^+ at a membrane potential of -60 mV. Outward current was recorded as downward deflection. Symbols “*” represent significant difference ($p < 0.05$).

Fig 6.
Illustration of UV absorption by anterior segment of the eye.

Fig 7.

Protection of corneal epithelial cells from UV irradiation-induced apoptosis by suppressing K⁺ channel activity. (A) Viability of rabbit and human corneal epithelial cells treated with UV irradiation or etoposide in the absence or presence of 4-AP (1 mM) blockade. Apoptotic cells were determined by EB/AO nuclear staining. Cell viabilities were determined 15–24 h after UV irradiation or etoposide (Eto) induction. (B) Effect of suppressing K⁺ channels on UV irradiation-induced rat corneal epithelium apoptosis detected with TUNEL. Normal rat corneas were stained with H-E staining for the histological structure (a). TUNEL assay was performed to detect cell apoptosis in the control cornea (b) and UV-exposed corneas in the absence (c) or presence (d) of 1 mM 4-AP. Symbols “*” represent significant difference ($p < 0.05$).

Fig 8.

Effect of suppressing K⁺ channel activity on UV irradiation-induced activation of JNK signaling pathway in rabbit corneal epithelial cells. (A) Time course of UV-induced JNK-1 activation. (B) Effects of selective K⁺ channel blockers on UV-induced JNK-1 activation. Channel blockers, 4-AP (1 mM), TEA (10 mM) and Ba²⁺ (5 mM), were applied to inhibit UV irradiation-induced JNK activation in these cells. Symbol “*” represents significant difference analyzed by one-way ANOVA and multiple comparison test, $p < 0.05$. (C) Comparison of inhibitory effect of 4-AP on UV irradiation-induced JNK phosphorylation and K⁺ channel activity in a dose-dependent manner.

Fig 9.

UV irradiation induced the phosphorylation of p53 in rabbit corneal epithelial cells. (A) Site-specific analysis of UV-irradiation-induced p53 phosphorylation. UV-irradiation-induced phosphorylations of p53 were analyzed with site-specific antibodies in Western blots. (B) Immunostaining of p53 and pp53ser15 induced by UV irradiation. Cellular expressions of p53 and pp53ser15 were detected by specific antibodies. Pictures were taken by a digital camera under Nikon fluorescent microscope (60 × power lens).

Fig 10.

Effect of suppressing Kv channel activity on UV irradiation-induced p53 phosphorylation. Rabbit corneal epithelial cells were pretreated with specific Kv channel blockers, including 4-AP (1 mM), Ba²⁺ (5 mM), α-DTX (200 nM) or BDS-I (400 nM), 30 min prior to UV irradiation. Phosphorylation of p53ser15 was analyzed by Western blot. Symbol “*” represents significant difference analyzed by one-way ANOVA and multiple comparison test, $p < 0.05$.

Fig 11.

Effects of 4-AP and caffeine on UV irradiation-induced phosphorylation of p53ser15 in rabbit corneal epithelial cells. (A) Effect of suppressing Kv channel activity with 4-AP on extended period of UV irradiation-induced phosphorylation of p53ser15. Phosphorylation levels of p53ser15 in the absence and presence of 1 mM 4-AP are plotted as a function of extended time of UV exposures. (B) Effect of caffeine on extended UV irradiation-induced phosphorylation of p53ser15. Phosphorylation levels of p53ser15 in the absence and presence of 5 mM caffeine are plotted as a function of extended time of UV exposures. For UV treatments, cells were exposed to UV irradiation with an extended time for 3, 5, 10, 15, 20, and 30 min (protocol B) instead of a standard protocol (protocol A) of 42 $\mu\text{J}/\text{cm}^2$. Phosphorylation levels of p53ser15 were detected by Western analysis in the absence and presence of 4-AP or caffeine.

Fig 12.

Hypothetical model of UV-induced signaling pathways in corneal epithelial cells. Future developments of our research are to explore mechanisms that underlie UV irradiation-induced Kv channel hyperactivity, cell shrinkage and scaffold kinase activation, resulting in JNK pathway activation and apoptosis.