Ultraviolet A exposure induces reversible disruption of gap junction intercellular communication in lens epithelial cells

DAYANG WU, JIANGYUE ZHAO, DI WU and JINSONG ZHANG

Department of Ophthalmology, The Fourth Affiliated Hospital of China Medical University, Eye Hospital of China Medical University, Key Lens Research Laboratory of Liaoning Province, Shenyang 110005, P.R. China

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Abstract. Gap junction intercellular communication (GJIC) is essential for the proper function of many organs including the lens. Disruption of GJIC can cause lens metabolic disorder and can induce cataracts. The purpose of this study was to investigate the signal transduction pathways involved in GJIC disruption following ultraviolet A (UVA) exposure in lens epithelial cells. Following exposure of human lens epithelial cells to UVA, connexin 43 (Cx43), the main component of gap junctions, was down-regulated at both the mRNA and protein levels. Furthermore, we observed that UVA exposure can increase protein kinase C activity and stimulate reactive oxygen species generation and lipid peroxidation. Using scrape load dye transfer technique, we found that the GJIC is compromised by UVA exposure. In addition, we demonstrated that UVA-induced modulation of GJIC was associated with p38 mitogen-activated protein kinase activation. More importantly, at non-lethal doses (10 J/cm^2), the UVA-induced GJIC disruption and the consequent alterations were reversible. Collectively, our data revealed a new signaling pathway in GJIC disruption following UVA exposure, suggesting that UVA-compromised gap junction activity may sensitize human lens to photoaging and cataract formation.

Introduction

Cataract is a clouding of the lens in the eye that affects vision and is the leading cause of blindness in the world. Cataract develops for a variety of reasons, such as long-term exposure to ultraviolet (UV) light, exposure to radiation, diabetes, alcohol, smoking, diet, and steroid use (1-3). Among these causative factors for cataract formation, UV-irradiation has received considerable attention as the eye needs light to provide vision and the lens receives UV-radiation. UV from natural sunlight is a form of non-ionizing radiation, within the wavelength range of 200-400 nm. According to biological functions, UV rays can be divided into UVC (200-280 nm), UVB (280-320 nm) and UVA (320-400 nm). In general, UVB rays are primarily absorbed by the cornea, while the majority of UVA rays are absorbed by the lens. A number of studies have shown that UV-radiation modulated reactive oxygen species (ROS) results in degradation, cross-linking, and aggregation of lens proteins and DNA damage, which is regarded as an important factor in cataractogenesis (4-6). However, the precise mechanism of UV-radiation-induced cataract formation is unclear.

Intercellular communication through gap junctions plays a critical role in the maintenance of tissue homeostasis (7,8). Gap junctions are intercellular channels that allow the direct exchange of low-molecular-weight (≤1000 Da) solutes such as ions Na^+, K^+, H^+ and cyclic nucleotides (9,10). By mediating the regulated intercellular diffusion of substances, gap junction intercellular communication (GJIC) plays an important role in the regulation of proliferation and differentiation, which is essential for the proper function of many organs, including the lens. Gap junctions are formed by a family of closely related proteins termed connexin (Cx). Six Cx subunits form a hemichannel. Each Cx has four transmembrane domains, one intracellular loop and two extracellular loops. The intra-cellular domains contain the COOH and NH_2 termini that control channel activities and determine its specificity (11,12). Based on the species and molecular weight, cDNA clone-derived Cx43, Cx46, Cx50, Cx56, Cx45.6, Cx44 and Cx49 were found to be expressed in the lens of human and vertebrate animals (13). Eckert *et al* developed a simple dye transfer method that can quantify intercellular dye diffusion through gap junctions in the mammalian lens to determine GJIC activity (14). They introduced two fixable fluorescent dyes, Lucifer yellow and rhodamine-dextran, into lens fiber cells via mechanical damage induced by removing the lens capsule. The lenses were incubated in the dye solutions for several minutes and subsequently examined by confocal microscopy. The high molecular weight dye rhodamine-dextran is impermeable through gap junctions and was used to identify the cells that have initially been loaded with tracer. The low molecular weight Lucifer yellow dye is gap junction permeable and acts as a tracer for diffusion through

Correspondence to: Dr Jinsong Zhang, Department of Ophthalmology, The Fourth Affiliated Hospital of China Medical University, Eye Hospital of China Medical University, Key Lens Research Laboratory of Liaoning Province, 11 Xinhua Road, Shenyang 110005, P.R. China
E-mail: cmu4h-zjs@126.com

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the gap junction channels. Using this method, they were able to determine a value for the absolute permeability of the gap junctions that connect the fiber cells in the lens cortex.

A number of studies have been performed to explore the possible mechanisms by which disruption of GJIC can induce cataract formation. Baruch *et al* have found that dysfunction of GJIC caused by Cx damage will lead to increased Ca^{2+} influx and will result in ion metabolic disorders. Accumulation of calcium in the lens subsequently activates calcium-dependent cysteine proteases that result in crystal protein aggregation and lens opacification (15). A previous study has also shown that GJIC activity can be inhibited under high glucose conditions in lens epithelial cells, suggesting a role for GJIC in permeable edema and ion metabolic disorders in the diabetic lens (16). Disruption of GJIC has been reported to promote lens epithelial cell apoptosis, which is a common pathological finding in many types of cataracts and important in the pathogenesis of cataract (17).

Phosphorylation of Cx43 can rapidly adjust the channel's open and closed status, and change the number of active channels by affecting Cx43 synthesis, transport, aggregation, disaggregation and degradation, thus ultimately regulating the function of the gap junction. UV and several kinases, include protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and casein kinase (CK) I and II have been found to regulate Cx43 phosphorylation, thus affecting the function of gap junction channels (18-20). Therefore, research on the UVA-induced phosphorylation of Cx43 and its relationship to the lens gap junction, as well as the effect of non-lethal UVA on the lens epithelial cells is of great significance. In this study, we aimed to investigate the signal transduction pathways involve in GJIC disruption following UVA exposure in lens epithelial cells. We found that UV disrupted GJIC activity through down-regulation of Cx43 expression, and this disruption was reversible in a p38-dependent manner.

Materials and methods

Cell culture. The human lens epithelial cell line SRA01/04 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air.

UVA-irradiation and cell viability assay. Confluent SRA01/04 cells were irradiated with UVA 365 nm at a dose of 10 J/cm^2 for 2 h. Cells were irradiated in PBS to avoid secondary toxicity due to culture medium modifications. After removal of PBS and addition of culture medium, irradiated cells were incubated at 37˚C in 5% CO_2 for various times. Unirradiated PBS-treated SRA01/04 cells were used as control.

Cell viability after UVA-irradiation was measured by a cell counting kit (CCK-8) assay (Beyotime Institute of Biotechnology, Haimen, China). SRA01/04 cells were suspended at a final concentration of 2000 cells/well and cultured in 96-well flat-bottomed microplates. After exposure to UVA for various doses, CCK-8 (10 μl) was added to each well and incubated for 1 h at 37˚C. Viable cells were counted by absorbance measurements at 450 nm (OD 450) using a microplate reader. The OD 450 value was proportionally and inversely correlated to the degree of cell apoptosis. All experiments were performed in triplicate and repeated at least three times.

RNA isolation and real-time RT-PCR. Total-RNA was isolated from treated and control cells at designated time points using the RNAsimple total-RNA kit (Tiangen Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's instruction. For reverse transcription, 2 μg of total-RNA was used to make 20 μl cDNA using the Superscript first strand synthesis kit system according to the manufacturer's protocol (Tiangen). Quantitative real-time PCR was carried out in 20 μl of reaction mixture containing 10 μl of 2X SYBR-Green Master mix (Tiangen), 0.5 μM of forward and reverse primers, and 1.5 μl template cDNA on a Exicycler 96 real-time PCR system (Bioneer, Daejeon, Korea) under the following conditions: 95˚C for 10 min, followed by 35 cycles of 95˚C for 10 sec, 58˚C for 20 sec and 68˚C for 40 sec. The primers for Cx43 were: 5'-TCTGAGTGCCTGAACTTGC-3' (sense) and 5'-ACTGACAGCCACACCTTCC-3' (antisense). Constitutively expressed β-actin mRNA was simultaneously assayed as an internal standard for sample normalization. All experiments were performed in triplicate and repeated at least three times. The expression of the Cx43 mRNA was calculated using the $2^{-\Delta\Delta CT}$ method as previously described (21).

Western blotting. Cells were lysed by sonication in a buffer containing 15 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 6% β-mercaptoethanol. Protein concentration was determined by the Bradford method. After incubation at 95˚C for 10 min, equal volumes of protein extracts were separated by 10% SDS-PAGE. The proteins were then transferred to an Immobilon™ PVDF membrane using a Western blotting apparatus. Membranes were blocked with 5% w/v non-fat dry milk and 0.1% v/v Tween-20 in PBS, and incubated with anti-Cx43 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-p38 (1:1000, Abcam, Cambridge, MA, USA), anti-p38 (1:1000, Abcam) or anti-GAPDH antibodies (1:5000, Abcam) overnight at 4˚C. Signals were visualized with a Phototope-HRP Western blotting detection kit (Cell Signaling Technology, Danvers, MA, USA).

PKC activity assay. After UVA-irradiation at a dose of 10 J/cm^2, SRA01/04 cells were incubated for different time points at 37˚C. PKC activity assay was performed using a PKC enzyme assay system (Genmed, Shanghai, China) according to the manufacturer's instruction. Results were analyzed with NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 340 nm 0 min - 340 nm 5 min, and data were normalized to the sample protein concentration.

Measurement of reactive oxygen species (ROS) and lipid peroxidation. After UVA-irradiation, cells were incubated at 37˚C, 5% CO_2 for various time points for repair. Cells were then incubated with the DCFH-DA probe (Beyotime) for 20 min. ROS was measured by flow cytometry using the ROS detection kit (Beyotime). Lipid peroxidation due to UVA-irradiation was measured as previously described (20). Briefly, SRA01/04 cells were plated in 60-mm petri dishes, 10^6 cells/dish, and incubated at 37˚C overnight. After UVA irradiation with 10 J/cm^2, cells

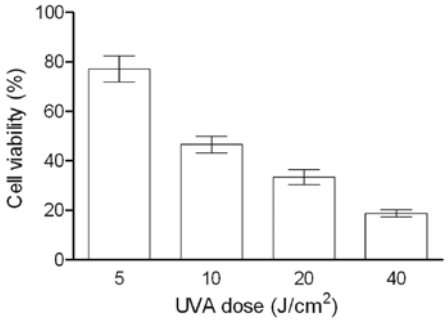

Figure 1. Cell viability after UVA (365 nm) irradiation of lens epithelial cells. SRA01/04 cells were irradiated with UVA at doses of 5, 10, 20 and 40 J/cm^2. Cell viability was measured by the CCK-8 assay. Absorbance was read at 450 nm. The means and standard deviation (error bar) from three experiments are depicted.

were incubated for various time points. Supernatants (200 μl) were collected and malondialdehyde (MDA) was measured with the thiobarbituric acid-reactive substances (TBARS) using the MDA assay kit (Beyotime). Data were normalized to the protein content of the same cells measured using the BCA kit (Beyotime). Data are expressed as the mean ± SD from 3 petri-dishes.

Scrape load dye transfer technique (SLDT). SLDT was used to assess GJIC activity as described previously (22,23). Briefly, after UVA-irradiation, the cells were washed three times with PBS, and 1.5 ml of PBS containing 0.05% Lucifer yellow fluorescent dye and 0.05% rhodamine-dextran was added. Several scrapes (cuts) were made on the monolayer using a surgical scalpel. The cells were incubated for 3 min at 37˚C and then rinsed three times with PBS. The cells were then fixed with 4% paraformaldehyde in PBS and photographed with a digital camera attached to a fluorescence microscope (Motic Electric Group Co., Ltd., Xiamen, China).

Statistical analysis. All data are expressed as means ± SD and raw data were analyzed by the unpaired Student's t-test using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). A P-value <0.05 was considered to be statistically significant.

Results

Lens epithelial cell line SRA01/04 sensitivity to UVA-irradiation. Confluent cells in 96-well plates were irradiated with UVA at doses ranging from 5-40 J/cm^2. Cell viability was measured by the CCK-8 assay and the values are shown in Fig. 1. At a dose of 5 J/cm^2, cell viability was ~80%. Cell viability remarkably decreased after an increase of the UVA dose to 40 J/cm^2. Cell viability was 46.4% at 10 J/cm^2 and this UVA dose was used in subsequent experiments.

Cx43 expression in UVA-irradiated lens epithelial cells. Cx43 mRNA levels were determined by means of real-time RT-PCR. When measured immediately after UVA-irradiation, Cx43 mRNA levels were significantly decreased compared to control cells (Fig. 2A). Cx43 mRNA levels were slightly increased 1 h after irradiation and remained higher 2-8 h thereafter. Cx43 mRNA levels returned to normal levels (those of control cells)

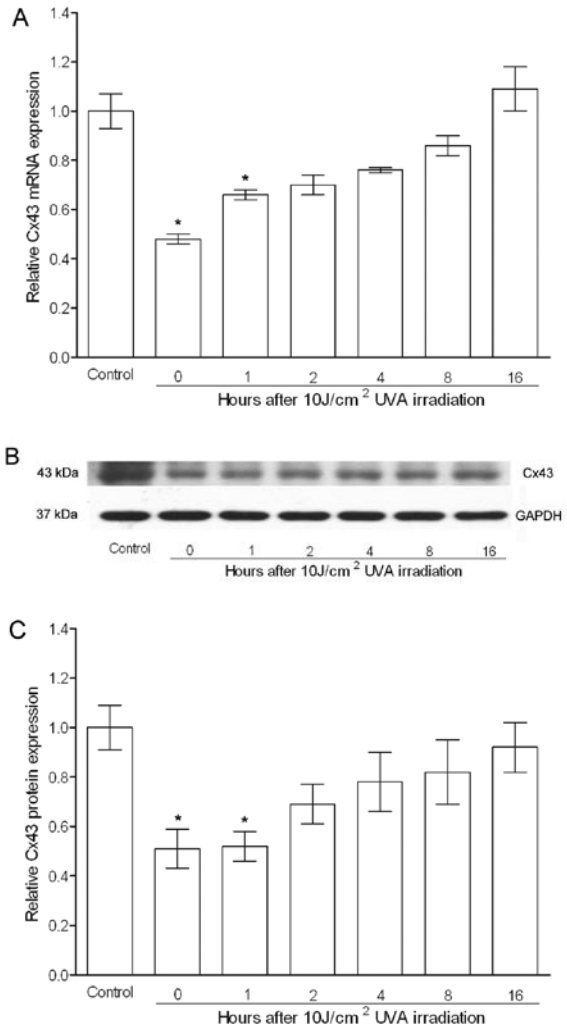

Figure 2. Cx43 expression in UVA irradiated lens epithelial cells. Cells were exposed to 10 J/cm^2 of UVA, and incubated for up to 16 h. (A) Cx43 mRNA levels were determined by real-time RT-PCR. Cx43 mRNA was significantly decreased after UVA-irradiation, but slightly increased 1 h after irradiation and remained higher thereafter. (B) Western blot analysis showed the same pattern of change in Cx43 protein levels after UVA-irradiation. Equivalent loading of the cell lysates was determined by anti-GAPDH immunoblotting. (C) Quantitative expression level of Cx43 protein was analyzed by measuring band density on the gel image. The means and standard deviation (error bar) from three experiments is depicted. *P<0.05 vs. control.

16 h later. The same pattern of change in Cx43 protein levels was observed after UVA-irradiation (Fig. 2B and C). After UVA-irradiation, Cx43 protein was significantly decreased. One hour after incubation, Cx43 protein gradually recovered and it returned to normal levels after 16 h incubation compared to unirradiated control cells. Thus, UVA reduces Cx43 expression at both the mRNA and protein levels, and this reduction is reversible at a UVA dose of 10 J/cm^2.

PKC activity in UVA-irradiated lens epithelial cells. UVA-irradiation stimulated PKC activity in SRA01/04 cells. PKC activity significantly increased within 4 h after UVA-irradiation compared to unexposed cells (Fig. 3). An increase >58% in PKC activity was observed immediately after UVA-irradiation relative to control cells. After 1 h of incubation, a slight decrease of PKC activity was observed, but it remained significantly higher. Two hours later, the PKC

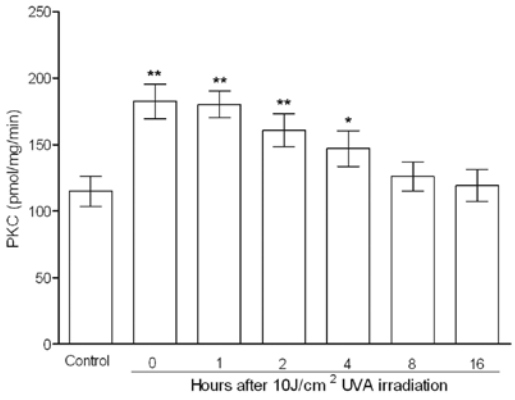

Figure 3. UVA-irradiation stimulated PKC activity in lens epithelial cells. SRA01/04 cells were exposed to 10 J/cm^2 UVA and incubated up to 16 h. PKC activity was measured by the PKC enzyme assay system. Compared to unirradiated control cells, PKC activity was significantly increased within 4 h after UVA-irradiation. Subsequently, PKC activity gradually decreased, and returned to normal levels by 16 h incubation. Data are the means ± SD of results from triplicate experiments. *P<0.05, **P<0.01 vs. control.

activity gradually decreased and was mostly recovered after 16 h of incubation, suggesting that UVA stimulated PKC activity is reversible.

UVA-irradiation generates ROS and stimulates lipid peroxidation. Flow cytometry analysis was performed to evaluate the ROS production by UVA-irradiation. UVA-irradiation generated a rapid and marked production of ROS (Fig. 4A), indicating the induction of oxidative stress by UVA. Following a 16 h incubation period, ROS levels decreased, suggesting that the balance of cellular stress was partially recovered (Fig. 4B). Similarly, lipid peroxidation measured by cellular MDA levels was rapidly increased after UVA-irradiation (Fig. 5). More than an 80% increase of MDA was observed immediately after UVA-irradiation, and it remained 60 and 40% higher compared to control cells after 1 and 2 h of incubation, respectively. In agreement with ROS production, at 16 h after UVA-irradiation, there was a partial recovery of lipid peroxidation.

UVA-irradiation causes a decrease of GJIC activity. GJIC activity was assessed using an SLDT assay. SRA01/04 cells were exposed to 10 J/cm^2 of UVA, and scrape loaded to determine their capacity to transfer Lucifer yellow. As illustrated in Fig. 6, in control cells, the fluorescent dye was detected in five to six layers, and the Lucifer yellow-coupled cells were located on both sides of the scrape line, suggesting that control cells possess a high dye transfer capacity. In contrast, 2 h after UVA-irradiation, the fluorescent dye was detected in only one to two layers, and the Lucifer yellow-coupled cells were mostly located in the scrape line, reflecting that UVA-irradiation induced a decrease in the dye transfer capacity of SRA01/04 cells. However, the UVA-induced GJIC decrease was gradually recovered after 4 h of incubation and returned to normal levels by 16 h of incubation.

p38 activation in response to UVA-irradiation in lens epithelial cells. p38 is an important modulator of the adaptive cell response to environmental stress (24). To investigate whether

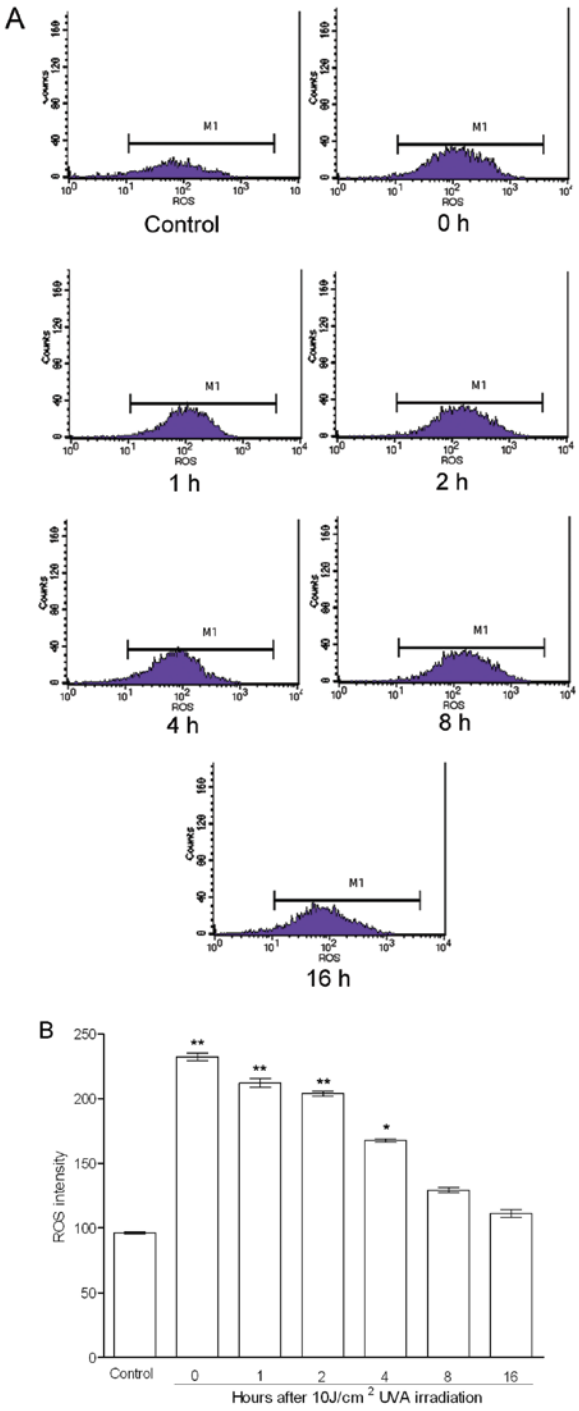

Figure 4. Generation of ROS after UVA-irradiation. After UVA-irradiation, SRA01/04 cells were incubated for up to 16 h for repair, and with the DCFH-DA probe for 20 min. (A) ROS was measured by flow cytometry using the ROS detection kit. (B) Quantitative analysis of ROS generation. Compared to unirradiated control cells, UVA generated a rapid and marked production of ROS within 2 h. After 4 h repair, the ROS level was decreased and return to normal after 16 h incubation. Data are the means ± SD of results from triplicate experiments. *P<0.05, **P<0.01 vs. control.

p38 activation was involved in UVA-irradiation, we exposed SRA01/04 cells with UVA at a dose of 10 J/cm^2, and incubated cells at 37˚C for up to 16 h. p38 activation was monitored by assessing the relative levels of p38 phosphorylation. The level of pho-p38 was increased within 2 h after UVA-irradiation, demonstrating that exposure to UVA in lens epithelial cells

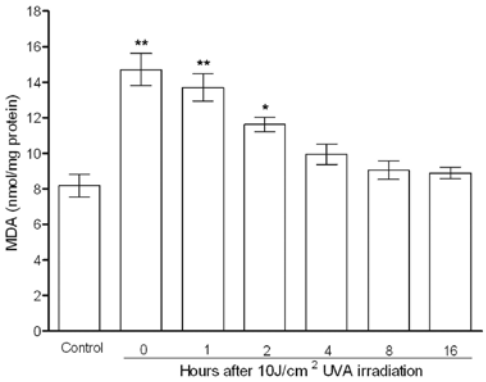

Figure 5. Lipid peroxidation after UVA-irradiation. SRA01/04 cells were plated in 60-mm petri dishes. After UVA-irradiation, cells were incubated for up to 16 h. Lipid peroxidation was analyzed by measuring the cellular MDA level using the MDA assay kit. More than 80% increase of MDA was observed immediately after UVA-irradiation, and it remained 60 and 40% higher, respectively compared to control cells after 1 and 2 h of incubation. After 16 h, the cellular MDA returned to normal levels. Data are the means ± SD of results from triplicate experiments. *P<0.05, **P<0.01 vs. control.

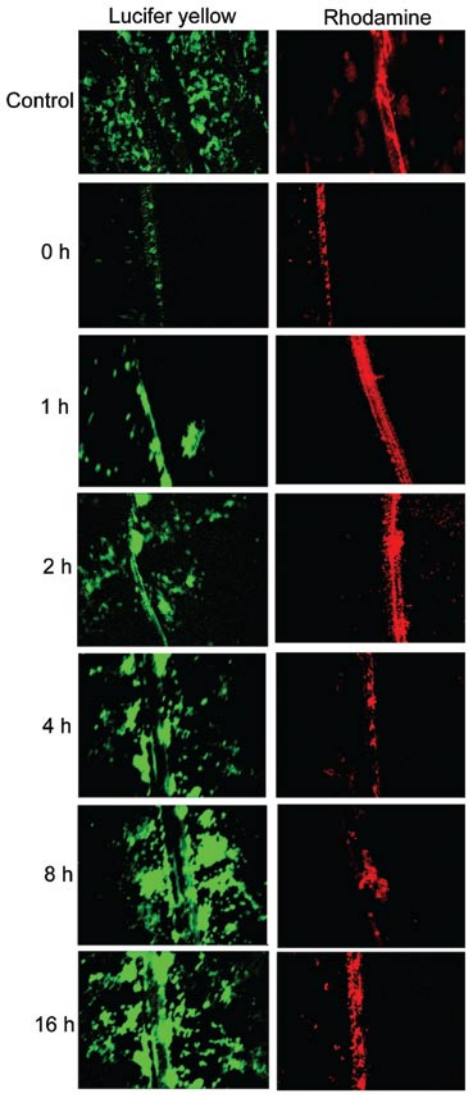

Figure 6. Gap junction intercellular communication activity in lens epithelial cells. Image analysis of SLDT assay. Cells were exposed to 10 J/cm^2 of UVA, and incubated for up to 16 h. The scrape cells were stained with 0.05% Lucifer yellow fluorescent dye and 0.05% rhodamine-dextran. The dye-coupled cells were observed under a fluorescence microscope.

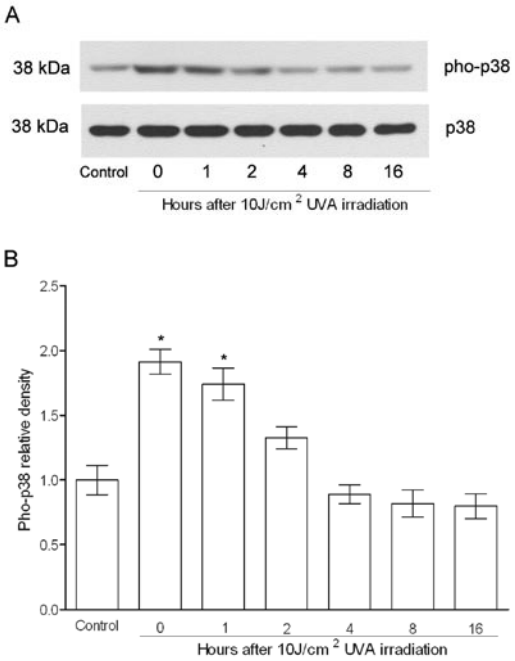

Figure 7. p38 activation in response to UVA. SRA01/04 cells were exposed to 10 J/cm^2 of UVA, and incubated for up to 16 h, and harvested. (A) The cell lysates were subjected to Western blotting to monitor phosphorylation of p38. (B) The relative levels of phosphorylation of p38 were assessed by normalization to total p38. The level of pho-p38 was increased within 2 h after UVA-irradiation. Over 4 h incubation, p38 phosphorylation level was gradually decreased, and returned to the same level as compared to unirradiated control cells 16 h later. The means and standard deviation (error bar) from three experiments are depicted. *P<0.05 vs. control.

resulted in p38 phosphorylation, as expected (Fig. 7). Notably, p38 phosphorylation level gradually decreased after 4 h incubation, and returned to the same level as compared to unirradiated control cells 16 h later, reflecting that the response to UVA stress in lens epithelial cells can be recovered (Fig. 7).

Discussion

Cataract is the most common pathology of the lens, and UVA-radiation is one of the important risk factor. Previous studies have shown that GJIC is a novel UVA target, and UVA-irradiation can disrupt the function of GJIC in human keratinocytes via p38 MAPK activation (19,20). UVA can induce various pathological changes in the lens epithelium. However, it has not been reported that whether UVA can induce the alteration of connexin expression in the lens epithelium under physiological conditions and whether these damages are reversible. In this study, we found that UVA (365 nm) can disrupt GJIC in human lens epithelial cells. This disruption was reversible and due to alteration of Cx43 expression, generation of ROS and lipid peroxidation stimulation, which is dependent on p38 activation by UVA.

Connexin phosphorylation is a common event with important implication in a variety of cellular process including gating of gap junction (25). In the lens, PKC, MAPK and CKI and II can catalyze connexin phosphorylation. Many PKC activators can increase the variety of cell Cx43 phosphorylation and reduced gap junction function (18). PKC can also directly phosphorylate Cx43 and decrease the gap junction

single-channel conductance and dye permeability in non-cardiac cells of the dye (26). In our study, we found a decrease in Cx43 at both the mRNA and protein levels and an increase in PKC activity induced by UVA, suggesting that through PKC-activated Cx phosphrylation, UVA may promote the degradation of Cx43 and result in a decrease in intercellular communication. Gap junction has been reported as a new target of UVA-irradiation in the epidermis (19,20). The gap junction can be regulated by Cx43 phosphorylation and dephosphorylation. Through this mechanism we demonstrated that lens epithelial cells exposed to UVA-irradiation display a reversible decrease in GJIC, supporting that Cx43 expression changes may be due to the regulation of the connexin phosphorylation status.

We observed that the reduction of GJIC activity was not due to a cytotoxic effect of UVA-irradiation, but rather due to ROS generation and lipid peroxidation in lens epithelial cells. We also demonstrated that this UVA-induced reduction was reversible at a dose of 10 J/cm^2. Our findings are consistent with previous studies (20). Inhibition of GJIC by UVA-irradiation has been reported to be associated with the deleterious effects of UVA on the human epidermis by generating ROS and compromising membrane stability (19). The role of ROS in GJIC is yet unclear, although it has been reported that oxidative stress can inhibit GJIC and antioxidants can reverse the down-regulation of GJIC (19,27,28). Our findings suggest that UVA activates ROS generation and lipid peroxidation which induce connexin degradation and down-regulation of its expression is a major cause of gap junction disruption following UVA injury.

MAPKs including ERK1/2, BMK-1 (ERK5), p38, JNK can phosphorylate Cx43 on the Ser279 and Ser282 sites, which cause a rapid and transient decrease of GJIC (29). Different kinases can act on the same site, and the same kinase can also act on different sites. Many kinases and phosphatases can directly or indirectly phosphorylate Cx43 and affect the function of gap junction channels. In addition, various kinases and phosphatases can also interact and form a complicated regulatory network. The p38 MAPK signaling pathway allows cells to interpret a wide range of external signals and respond appropriately by generating a plethora of different biological effects (30). Phosphorylation and activation of p38 kinase is related to a cellular stress condition and its activity contributes to the regulation of growth arrest, differentiation and apoptosis in response to a variety of cellular stresses (31,32). Previously, UVA-induced GJIC down-regulation in human keratinocytes has been reported to be involved in p38 activation-dependent Cx43 phosphorylation and degradation (19). In this study, we showed the phosphorylation of p38 after UVA-irradiation in lens epithelial cells, suggesting that UVA-induced p38 MAPK activation also plays an important role in modulation of GJIC in lens epithelial cells.

In conclusion, we found that UVA can disrupt the function of GJIC in lens epithelial cells. We demonstrated that this UVA-induced GJIC disruption is mediated by alteration of Cx43 expression, generation of ROS and lipid peroxidation stimulation, which is reversible and dependent on p38 activation. Future studies will focus on the experimental modulation of Cx43 expression and phosphorylation, and its effects on the signaling pathways in cataractogenesis.

Acknowledgements

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