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MEK/ERK pathway mediates UVB-induced AQP1 downregulation and water permeability impairment in human retinal pigment epithelial cells

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Abstract. Aquaporins (AQPs) are a family of 13 small (~30 kDa/monomer), hydrophobic, integral membrane proteins. AQPs are expressed in various epithelial and endothelial cells involved in fluid transport. Here, we demonstrated for the first time that AQP1 is expressed in cultured human retinal pigment epithelial (RPE) cells (ARPE-19 cell line). Ultraviolet radiation (UVB) and H_2O_2, two major factors causing RPE cell damage, induced AQP1 downregulation which was mediated by MEK/ERK activation. UV and H_2O_2 as well as AQP1-specific siRNA knockdown impaired water permeability of ARPE-19 cells. Notably, pretreatment with all-trans retinoic acid attenuated UV- and H_2O_2-induced AQP1 downregulation and water permeability impairment. Considering that water permeability is involved in multiple functions of RPE cells such as cellular junction formation, fluid or protein exchange and barrier formation, our data elucidated a novel mechanism through which UV radiation and oxidative stress induce eye cell damage. Our results further support the notion that all-trans retinoic acid might be useful for protection against UV or oxidative stress-induced eye cell damage.

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Introduction

The role of UV in skin carcinogenesis has been extensively studied because of the large exposure of this organ. The eye is another directly exposed organ; however, very little is known about the effect of UV exposure on the retina (1). One previous study described the *in vitro* effect of UV-C irradiation on lens α-crystallin, a protein thought to play a role in maintaining lens transparency (2), whereas other studies have analyzed signaling pathways associated with corneal epithelial cell and retinal pigment epithelial cell apoptosis (1,2). Components of UV are capable of reaching the human retina, as shown by a structural study of the rat retina exposed to UV. Both UV-B and UV-C produce alterations which affect the function of the retina (3). However, the detailed mechanisms involved in UV-induced eye damage have not been fully studied.

Retinal pigment epithelial (RPE) cells are highly polarized cells. Their integrity is critical for the maintenance of neural retina functions. In healthy subjects, RPE cells have a limited potential of proliferation associated with growth and age, while in uncontrolled RPE cells proliferation may contribute to retinal diseases such as proliferative vitreoretinopathy (PVR) (4,5). On the other hand, RPE is thought to be the prime early target for age-related macular degeneration (AMD), which involves RPE cell death and atrophy of the photoreceptors (6). Furthermore, RPE cells are also major targets for UV radiation or oxidative stress-induced damage (1,7,8), which is also a major cause of AMD (6). Studies performed in RPE fields used a spontaneously arising human RPE cell line, or ARPE-19, as a cellular model (1,9). ARPE-19 cells exhibit a polarization, capable of tight junction formation (9).

Aquaporins (AQPs) are a family of small (~30 kDa/monomer), hydrophobic, integral membrane proteins which are expressed widely in the animal and plant kingdoms, with 13 members having thus far been identified in mammals (10,11). AQPs are expressed in various epithelia and endothelia involved in fluid transport, as well as in cell types that were originally thought not to carry out fluid transport (11). AQP1 is thus far the only AQP that is expressed in RPE

(12,13). AQP1 mediates osmolarity-driven water transport across epithelial cells linking the proximal tubules and descending thin limbs of Henle's loop, and across endothelial cells of the descending vasa recta (9). Studies in AQP1-null mice have demonstrated that AQP1 mediates osmolarity-driven water transport across the epithelial barrier in serosal membranes such as the pleura and the peritoneum, in lung microvessels, and in the cornea (14). AQP1 in RPE *in vivo* probably contributes to the efficient trans-epithelial water transport across RPE, maintains retinal attachment, and prevents subretinal edema (1).

Given that UV radiation and oxidative stress are major causes of RPE cell damage and that AQP1 plays an important role in maintaining the normal function of RPE (13), we conducted this study to test the effects of UV and oxidative stress on AQP1 expression and function. We demonstrated for the first time that UV radiation and H_2O_2 treatment down-regulates AQP1 expression in ARPE-19 cells. We found that ROS-mediated MEK/ERK activation is involved in this downregulation process. AQP1-mediated water permeability is also impaired by UV and H_2O_2 treatment. Notably, all-trans retinoic acid (atRA) pre-treatment attenuated UV- and H_2O_2-induced AQP1 downregulation and water permeability impairment. Since water permeability is involved in multiple functions of RPE cells such as cellular junction formation, fluid or protein exchange and barrier formation (13,15-17), our study elucidated a novel mechanism through which UV radiation and oxidative stress induce eye cell damage. Our data also support the notion that all-trans retinoic acid might be useful for protection against UV- or oxidative stress-induced eye cell damage.

Materials and methods

UV light apparatus. The UV-irradiation apparatus used in this study was previously described (18-21). Before UV irradiation, cells were washed with 1 ml pre-warmed PBS buffer. Cells were irradiated at the desired intensity without a plastic dish lid. After UV irradiation, cells were returned for incubation in basal medium with treatments for indicated time periods.

Chemicals and reagents. Rabbit anti-human aquaporin 1 (AQP1) was obtained from Chemicon (Temecula, CA) (AB3065), and anti-p-MEK, anti-MEK, anti-p-ERK, anti-ERK were obtained from Cell Signaling Technology (Danvers, MA). Monoclonal mouse anti-ß-actin, all-trans retinoic acid (atRA) and n-acetyl-l-cysteine (NAC) were obtained from Sigma (St. Louis, MO). U0126, PD98059, JNKi and SB203580 were from CalbioChem (San Diego, CA).

Cell culture. Human retinal pigment epithelial (RPE) cells (ARPE-19 cell line) were a gift from the laboratory of Dr Fu Shang (22). ARPE-19 cells were maintained in a DMEM medium supplemented with 10% fetal bovine serum, penicillin/streptomycin and 4 mM L-glutamine, in a CO_2 incubator at 37˚C.

Western blot analysis. As reported previously (19,20,23), 20 μg protein from cells with different treatments was separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA). After blocking, membranes were incubated with specific antibodies. Antibody binding was detected using an enhanced chemiluminescence (ECL) detection system (GE, Piscataway, NJ) by autoradiography with Hyperfilm.

Measurement of water permeability. The water permeability was measured using a well established method as described previously (20). The coverslips with ARPE-19 cells were mounted in a closed perfusion chamber on the stage of a Zeiss fluorescence microscope. The cells were loaded with calcein, and the water permeability measurement was conducted (see details below). First, the cells were loaded with calcein by a 5-min exposure to 20 μM calcein-acetoxymethyl ester (calcein-AM, Invitrogen, Carlsbad, CA) in 300 mosM PBS. The loading of the cells with calcein was performed at 30˚C for 10 min. The subsequent water permeability measurements were carried out at 10˚C to decrease the water diffusion through the lipid bilayer of the plasma membrane. The cells were then perfused with cold 300 mosM PBS for 5 min, then, the perfusate was switched to 150 mosM PBS at the same temperature. Fluorescence intensity of perfused cells was recorded every 2 min (0, 2, 4 and 6 min) with excitation at 488 nm and emission at 515-525 nm (green) before and after the solution switch. The series of images were analyzed by measuring the time course of the calcein fluorescence in cytoplasmic regions of individual cells, and the fluorescence intensity was quantified. The half life of deduction of fluorescence intensity was used as a comparable indicator of water permeability. At least 100 cells in 6 random views were quantified for relative fluorescence intensity.

Reactive oxygen species (ROS) detection. ROS generation was detected by FACS analysis as described previously (20). Briefly, cultured ARPE-19 cells were loaded with 1 μM of fluorescent dye dihydrorhodamine (DHR) 2 h before treatment, which reacts with ROS in cells and results in a change of fluorescence. After being treated with UV with or without reagents for the desired time, ARPE-19 cells were trypsinized, suspended in ice-cold PBS and fixed in 70% ethyl alcohol at -20˚C. Changes in fluorescence in the drug-treated cells were quantified by FACS analysis. Induction of ROS generation was expressed in arbitrary units.

RNA interference (RNAi) experiments. Custom SMART pool® RNAi duplexes for AQP1 were from Dharmacon Research (Lafayette, CO). As described previously (24), ARPE-19 cells were cultured in complete medium for 4 days. Cells ($50x10^4$) were seeded into a 6-well plate 1 day prior to transfection and cultured to 60-70% confluence the following day. Lipofectamine™ LTX (6.25 μl) together with 2.5 μl PLUS™ Reagent (Invitrogen, Indianapolis, IN) was diluted in 90 μl of DMEM for 5 min in room temperature. Then, 8 μl AQP1 siRNA was mixed with DMEM containing Lipofectamine together with PLUS reagent and incubated for 30 min at room temperature. Finally, the complex was added to the well containing 2 ml medium with the final AQP1 siRNA concentration of 100 nM. AQP1 protein expression was determined by Western blot analysis 48 h after transfection.

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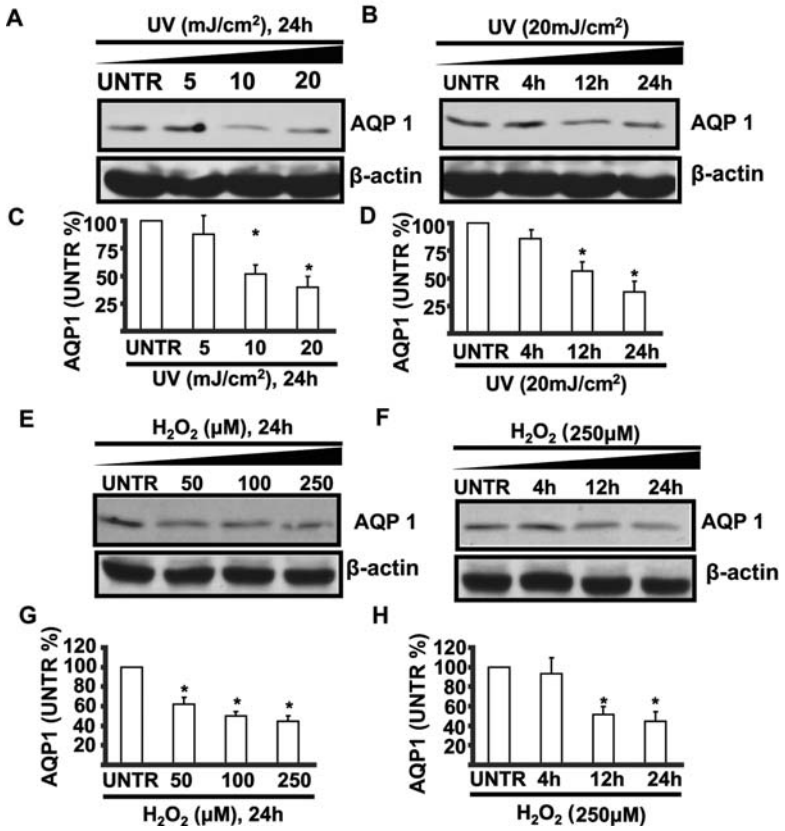

Figure 1. UV and H_2O_2 induce AQP1 downregulation in cultured human retinal pigment epithelial ARPE-19 cells. Cells were treated with UV (5, 10 and 20 mJ/ cm^2), or H_2O_2 (50, 100, 250 μM) and harvested at 24 h as shown in A and C, E and G (respectively), or treated with UV (20 mJ/cm^2) and harvested at 4, 12 and 24 h or H_2O_2 (250 μM) and harvested at 4, 12, 24 h as shown in B and D, F and H (respectively). AQP1 was analyzed by Western blot analysis. Data represent the mean ± SE of three independent experiments. *P<0.05 compared with the untreated (UNTR) group (lane 1).

Statistical analysis. The values in the figures were expressed as the means ± standard error (SE). The figures in this study are representative of more than 3 different experiments. Statistical analysis of the data between the control and treated groups was performed by the Student's t-test. Values $p<0.05$ were considered statistically significant.

Results

UV and H_2O_2 downregulate AQP1 expression in cultured human retinal pigment epithelial ARPE-19 cells. We studied whether UV and H_2O_2 affected AQP1 expression in cultured ARPE-19 cells. As shown in Fig. 1A and C, UV induced AQP1 downregulation in a dose-dependent manner, and AQP1 started to decrease at 10 mJ/cm^2 of UV and was most obvious at 20 mJ/cm^2 of UV. UV also induced AQP1 downregulation in a time-dependent manner. AQP1 started to decrease at 12 h and was most obvious at 24 h after 20 mJ/cm^2 of UV radiation (Fig. 1B and D). Similar results were also noted in H_2O_2-treated ARPE-19 cells (Fig. 1E-H). Collectively, our data demonstrated that UV and ROS induced AQP1 downregulation in cultured human retinal pigment epithelial ARPE-19 cells.

MEK/ERK inhibitors and antioxidants inhibit UV- and H_2O_2-induced AQP1 downregulation in cultured human retinal pigment epithelial ARPE-19 cells. We studied the signal pathways involved in this UV-induced AQP1 downregulation. Cultured ARPE-19 cells were pretreated with JNKi (JNK inhibitor), SB202580 (a p38 kinase inhibitor), PD98059 and U0126 (MEK/ERK inhibitors) followed by UV radiation. The results showed that MEK/ERK inhibitors PD98059 and U0126 inhibited UV-induced AQP1 downregulation, while the other inhibitors had little effect (Fig. 2A and B). Furthermore, PD98059 and U0126 also inhibited H_2O_2-induced AQP1 downregulation (Fig. 2C and D). Since ROS production plays critical roles in UV-induced cellular responses (25), we next tested the role of antioxidants in UV- and H_2O_2-induced AQP1 downregulation. We found that pretreatment with N-acetyl cysteine (NAC) largely inhibited UV- and H_2O_2-induced AQP1 downregulation (Fig. 2E and F). As expected, NAC pretreatment inhibited UV- and H_2O_2-induced ROS production (Fig. 2G). Furthermore, NAC also inhibited UV-induced ERK activation (Fig. 2H and I), which suggests that ROS-mediated MEK/ERK activation is involved in UV-induced AQP1 downregulation.

All-trans retinoic acid (atRA) attenuates UV- and H_2O_2-induced AQP1 downregulation in cultured human retinal pigment epithelial ARPE-19 cells. Since our previous studies demonstrated that atRA inhibits AQP3 downregulation in cultured skin keratinocytes (19), we next tested the effect of

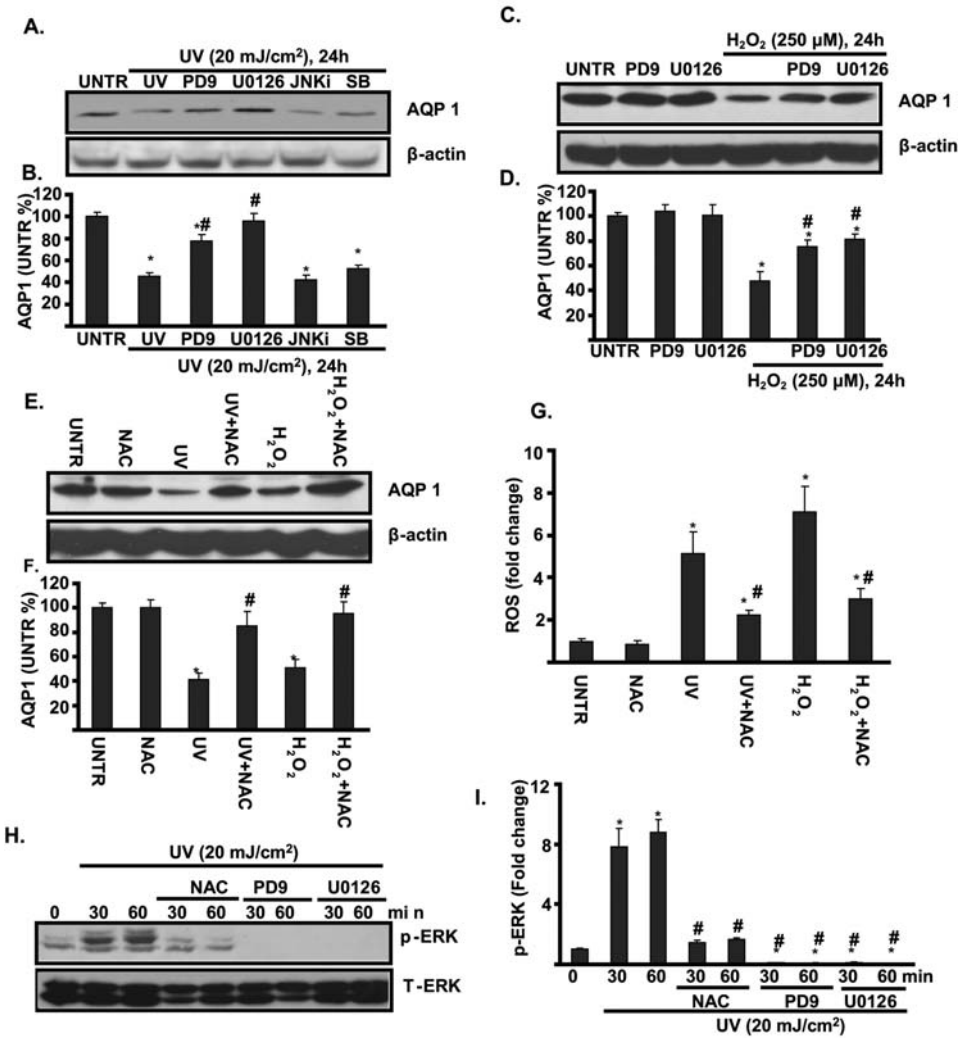

Figure 2. MEK/ERK inhibitors and antioxidant NAC inhibit UV/H_2O_2-induced AQP1 downregulation. ARPE-19 cells were pretreated with MEK/ERK inhibitor PD98059 (PD9, 1 μM) or U0126 (1 μM), JNK inhibitor (JNKi, 1 μM) and p38 inhibitor SB203580 (SB, 1 μM) for 1 h, followed by UV (20 mJ/cm^2) radiation (A and B) or treatment with H_2O_2 (C and D), and cells were harvested at 24 h for AQP1 detection by Western blotting as mentioned above. ARPE-19 cells were pretreated with or without N-acetyl cysteine (NAC, 400 μM) for 1 h, followed by UV radiation (20 mJ/cm^2) or H_2O_2 (250 μM). AQP1 expression was detected by Western blotting after 24 h (E and F), and ROS production was detected at 2 h (G). ERK phosphorylation in cells with indicated treatments was detected by Western blotting (H and H). Data represent the mean ± SE of three independent experiments. *P<0.05 with untreated (UNTR) group (lane 1); $^\#$P<0.05 with UV- or H_2O_2-treated group.

atRA on UV-induced AQP1 downregulation in cultured ARPE-19 cells. Pre-treatment with atRA for 1 h, dose-dependently rescued UV- (Fig. 3A and B) or H_2O_2- (Fig. 3C and D) induced AQP1 downregulation. Furthermore, we found that pre-treatment with atRA remarkably reduced UV- or H_2O_2-induced ERK activation (Fig. 3E). Notably, atRA alone upregulated AQP1 expression in cultured ARPE-19 cells in both a dose- (Fig. 3F and G) and time- (Fig. 3H and I) dependent manner. These results indicate that atRA, which alone induced AQP1 upregulation, attenuated UV- or H_2O_2-induced AQP1 downregulation in ARPE-19 cells.

All-trans retinoic acid attenuates UV- and H_2O_2-induced impairment of water permeability in cultured human retinal pigment epithelial ARPE-19 cells. We tested the functional results of AQP1 downregulation by focusing on water permeability. First, as shown in Fig. 4A and B, AQP1-specific siRNA successfully knocked down AQP1 expression in ARPE-19 cells. Second, while atRA, which alone up-regulated AQP1 expression (as shown in Fig. 3F and H), enhanced water permeability, UV radiation and H_2O_2 treatment as well as AQP1 siRNA knockdown impaired water permeability in ARPE-19 cells (Fig. 4C and D). Furthermore, pre-treatment with atRA attenuated UV- (Fig. 4E) and H_2O_2- (Fig. 4F) induced decrease of water permeability.

Discussion

We demonstrated for the first time that AQP1 is functionally expressed in cultured human retinal pigment epithelial cell line ARPE-19, which provides a cellular model by which to examine the function of AQP1 in RPE. We found that UV radiation and H_2O_2 treatment downregulated AQP1 expression (Fig. 1) and impaired water permeability (Fig. 4) in cultured

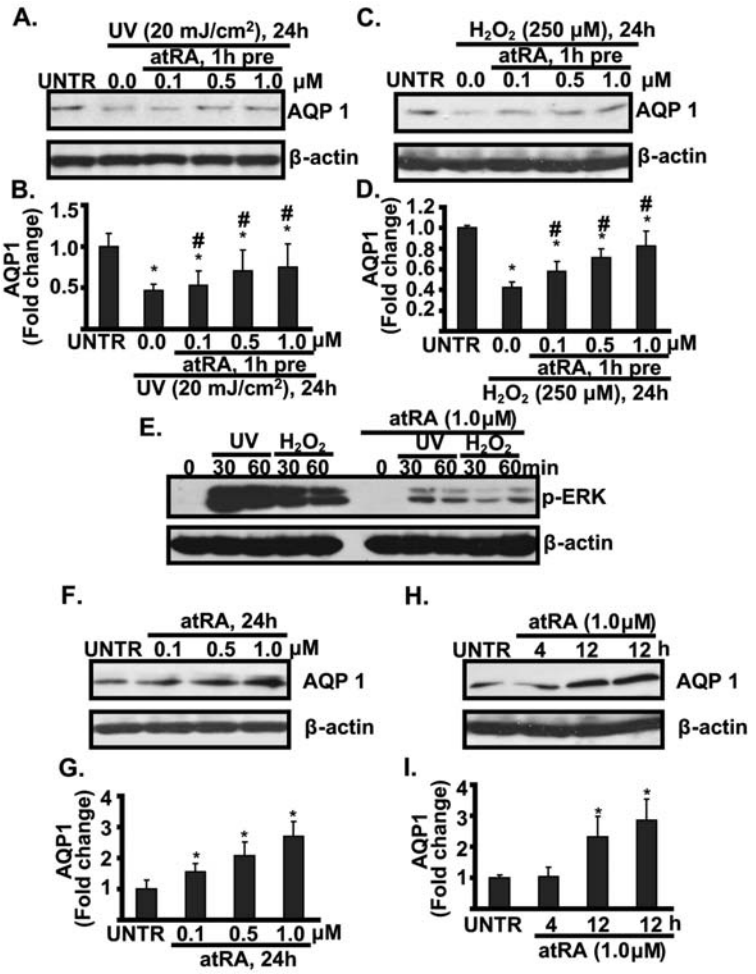

Figure 3. All-trans retinoic acid (atRA) attenuates UV- and H_2O_2-induced AQP1 downregulation. ARPE-19 cells were pre-treated with different doses (0.0, 0.1, 0.5 and 1.0 μM) of atRA for 1 h, cells were then washed in PBS 3 times before being treated with 20 mJ/cm^2 of UV (A and B) or H_2O_2 (C and D) and then incubated for 24 h in basic DMEM medium (without atRA), AQP1 expression was detected by Western blot analysis. Cells with or without 1 h of atRA (1.0 μM) pretreatment were treated with UV or H_2O_2, and then incubated for 30 and 60 min, and ERK phosphorylation was detected by Western blot analysis (E). Cells were treated with indicated doses of atRA for 24 h (F and G), or treated with 1.0 μM of atRA for indicated time points (H and I). AQP1 expression was detected by Western blotting.

retinal pigment epithelial ARPE-19 cell line. Mechanistic studies showed that ROS-mediated ERK activation was involved in this process (Fig. 2). Notably, we observed that all-trans retinoic acid, which alone induced AQP1 upregulation, attenuated UV- and H_2O_2-induced AQP1 downregulation and water permeability impairment (Figs. 3 and 4).

Water movement across the plasma membrane can occur via two pathways: by diffusion through the lipid bilayer (26) and via membrane-inserted water channels (aquaporins) (27-29). AQP1 is so far the only known aquaporin expressed in RPE. It has been demonstrated that AQP1 is functionally expressed in RPE, and AQP1 facilitates water movement across RPE monolayers in an expression-dependent manner in these two complementary model systems, and the expression of AQP1 by RPE *in vivo* probably contributes to the efficient trans-epithelial water transport across RPE, maintains retinal attachment, and prevents subretinal edema (13). Our study demonstrated that UV and oxidative stress actually down-regulated AQP1 expression and decreased water permeability which might be another mechanism to explain the impaired RPE function after UV or oxidative stress (1,30,31), since water permeability is involved in multiple important functions in RPE cells such as cellular junction formation, fluid or protein exchange and barrier formation (13,15-17).

Our study also demonstrated that pre-treatment with all-trans retinoic acid attenuated UV- and oxidative stress-induced AQP1 downregulation and water permeability impairment (Figs. 3 and 4). This observation is consistent with our previous study (19) and other reports (32) showing that atRA has similar effects on AQP3. Our data further suggest that inhibition of ERK activation is involved in the effects of atRA against UV- or oxidative stress-induced AQP1 down-regulation, which is also consistent with previous research using cultured skin keratinocytes (33). However, this does not exclude the possibility that mechanisms besides ERK inhibition might also be involved in the effect of atRA.

In conclusion, our study elucidated the mechanism through which UV and oxidative stress induce eye cell damage, namely the antioxidant-sensitive MEK/ERK pathway leading to downregulation of AQP1 and impairment

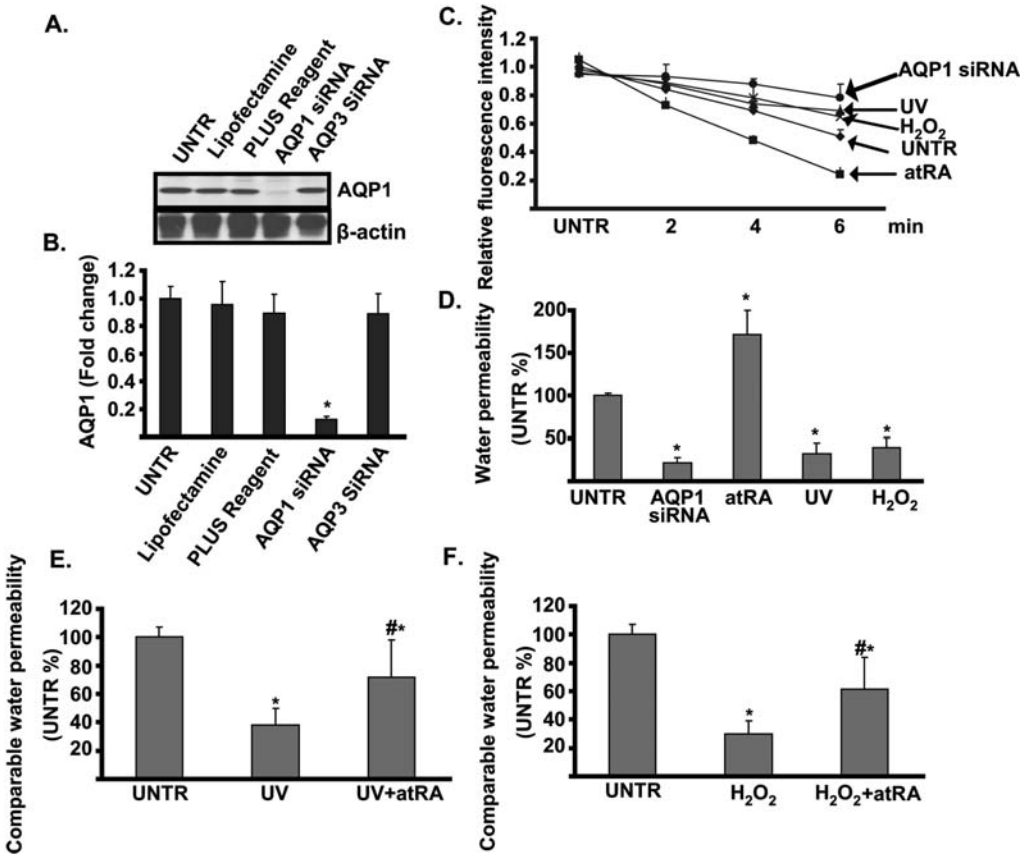

Figure 4. All-trans retinoic acid attenuates UV- and H_2O_2-induced impairment of water permeability in cultured human retinal pigment epithelial cells. ARPE-19 cells were treated with AQP1 siRNA, control siRNA (AQP3 siRNA) or transfection control for 48 h. AQP1 expression was detected by Western blot analysis (A and B). The water permeability of ARPE-19 cells with indicated treatments [untreated (UNTR), AQP1 siRNA for 48 h, 20 mJ/cm^2 of UV or 250 μM of H_2O_2 for 24 h, 1 μM of atRA for 24 h] was analyzed by the methods described previously, and the relative intensity of calcein fluorescence in cytoplasmic regions of individual cells after transfections or treatment was quantified in (C). The half-life of deduction of fluorescence intensity was used as a comparable indicator of water permeability (D). ARPE-19 cells without or with atRA (1 μM) pre-treatment were treated with UV (20 mJ/cm^2) or H_2O_2, and comparable water permeability was detected by the methods mentioned previously (E and F). For the cell water permeability experiments, at least 100 cells in 6 random views were quantified for relative fluorescence intensity. *P<0.05 vs UNTR; #P<0.05 vs UV-treated group.

of water permeability in ARPE-19 cells. Our data support the notion that all-trans retinoid acid and antioxidants might be useful for protection against UV- and oxidative stress-induced eye cell damage.

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