Beta-adrenergic agonist protects retinal pigment epithelium against hydroxycholoroquine toxicity *via* cAMP-PKA signal pathway

Ruihua Zhang[1], Dan–Ning Hu[1,2], Richard Rosen[1,2]

[1]Eye and Vision Research Institute, Department of Ophthalmology, Icahn School of Medicine at Mount Sinai, New York 10029, USA
[2]Department of Ophthalmology, New York Eye and Ear Infirmary of Mount Sinai, New York 10003, USA
Correspondence to: Richard Rosen. Department of Ophthalmology, New York Eye and Ear Infirmary of Mount Sinai, 310 East 14 Street, Suite 319SB, New York 10003, USA. rrosen@nyee.edu
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Abstract

● **AIM:** To test our hypothesis that activation of protein kinase A (PKA) signal pathway by β-adrenergic agonist plays an important role in the protecting of cultured retinal pigment epithelial (RPE) cells against the hydroxychloroquine (HCQ) toxicity.

● **METHODS:** Cultured human RPE cells were treated with 1) HCQ, 2) HCQ with salbutamol (a β2-adrenergic receptor agonist), and 3) HCQ with salbutamol and a PKA inhibitor, and compared these to 4) untreated cells (controls). After treated for 24h, cell vacuolation, cells viability, PKA and PKA kinase activity levels were determined by the measurement of the size of vacuoles using Image J software, the cell counting with a dye-exclusion testing, Western blot and PKA kinase detection, respectively.

● **RESULTS:** Cell vacuolation and cell death of cultured RPE cells were significantly increased by the treatment of HCQ. Salbutamol significantly elevated PKA and PKA activity levels and this was associated with the inhibition of the vacuolation and cell death. The PKA inhibitor significantly decreased the PKA levels and eliminated the protective effects of salbutamol on HCQ-treated RPE cells.

● **CONCLUSION:** The PKA pathway plays an important role in the protective effects of β2-adrenergic agonist on the RPE cells against HCQ toxicity. These findings reveal a novel potential strategy against HCQ retinopathy by treatment with PKA activating medications.

● **KEYWORDS:** hydroxycholoroquine; retinal pigment epithelial cells; retinopathy; protein kinase A; vacuolation

INTRODUCTION

Chloroquine (CQ) and hydroxycholoroquine (HCQ) were introduced early in 20 Century for the treatment and prophylaxis of malaria. In 1950s, their use was further expanded as potent agents for the treatment of rheumatic diseases. CQ came into use in 1953, followed by HCQ in 1955[1]. Since then, these two medications have been widely prescribed for the treatment of non-organ specific autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and mixed connective tissue disorders with good therapeutic effects and minimal immediate side effects[1-3]. Long-standing maintenance therapy is usually required. However, long term intake of CQ and HCQ poses a significant risk for development of retinopathy and severe vision loss. CQ has been gradually replaced by HCQ for the clinical uses due to the expanded total dosage safety and fewer side effects[1,3]. The incidence of CQ retinopathy was reported as 3%-10% in patients receiving CQ treatment[3]; whereas the incidence of HCQ retinopathy appeared lower, in the range of 0.5%-2% in various reports[1-2]. However, a landmark epidemiological study of 2361 patients found that the overall prevalence of HCQ retinopathy was 7.5% in patients who used HCQ continuously for more than 5y, and increased to around 20% after 20y of therapy. This suggests that HCQ retinopathy is more common than previously recognized[4]. Risk factors relevant to the occurrence of retinopathy include mainly dosage and duration of treatment. High daily and cumulative dosages and long period of treatment lead to a higher incidence of retinopathy[1-2,4].

Early signs of CQ and HCQ retinopathy are the appearance of subtle paracentral or parafoveal scotomas, which are usually not noticed by the patients. Gradually, a pigment retinopathy develops, with macular pigment mottling and loss of the foveal reflex, followed by a ring of depigmentation around the fovea

(bull's eye maculopathy). Subsequently, a central scotoma and loss of visual acuity occur. As the disease progresses, peripheral pigmentary irregularity and bone spicule formation may be associated with loss of both central and peripheral vision[1-3].

There is no proven medical therapy in HCQ toxicity other than stopping the drug. However, the retinopathy may continue to progress for some years even after cessation of therapy[1-2,5]. Therefore, the search for an antidote to this toxicity is required given the widespread adoption of HCQ as a long-term solution for connective tissue disease.

The retinal toxic effects of HCQ are mainly due to reduction in acidity of the lysosomes, which results in inhibition of lysosomal enzyme activity, blockage of the autophagy process, increasing vacuolation, and cell death[6-10]. This toxic effect affects mainly the retinal pigment epithelial (RPE) cells, ultimately damaging of the photoreceptors and resulting in severe vision loss[2-3,5,10]. There have been a number of studies exploring of agents that might recover lysosome function lost due to other toxic substances[8-9].

However, there has been no previous report of any systematic search of anti-HCQ toxicity agents using *in vitro* RPE cells models. In 2016, we developed an *in vitro* model using cultured human RPE cells which demonstrates the most important features of HCQ-induced damage, vacuolation in the cytoplasm with inhibition of cell growth at moderate dosages of HCQ, and cell death at higher doses of HCQ. This model is useful for exploring potential antidotes for the treatment of HCQ retinopathy[11-12].

Our previous studies demonstrated that β1- and β2-adrenergic receptor agonists, dopamine receptors 1, 5 agonists and purinergic receptor agonists significantly protected the RPE cells against the HCQ toxic effects[12]. All of these agents have cyclic adenosine monophosphate (cAMP)-elevating effects and our previous studies documented that β-adrenergic agonists stimulated cell proliferation and melanogenesis of uveal melanocytes *via* the cAMP signal pathway[13]. The main downstream signal of the cAMP pathway is protein kinase A (PKA).

The adrenergic agonist we selected in the present study was salbutamol (a adrenergic β2-receptor agonist), which has showed significant protection of RPE cells against HCQ toxicity *in vitro*[12]. In our previous studies, three different dosages of salbutamol have been tested (10^{-6}, 10^{-5} and 10^{-4} mol/L). All of these dosages showed significant protective effects. The highest dosage (10^{-4} mol/L) showed similar protective effects as 10^{-5} mol/L, which were slightly superior to the lowest dosage (10^{-6} mol/L)[12]. Therefore, the 10^{-5} mol/L dosage was selected in the present study. The interval between the addition of salbutamol and HCQ has been tested at four different intervals, salbutamol added simultaneously with HCQ or prior to HCQ at 30min, 1, 2 and 3h. We found that salbutamol added to the culture 2h before HCQ revealed the best protective effects[12]. Therefore, this interval was used in the present study. We hypothesize that activation of PKA signal pathway by β2-adrenergic agonists plays an important role in the protecting of RPE cells against the HCQ toxicity.

The role of activation of PKA signal pathway in the protection of RPE cells against HCQ toxicity has not been previously reported. The purpose of this study was to measure PKA and PKA kinase activity levels[14] and cell toxic effects in RPE cells treated with HCQ, with or without the addition of β-adrenergic agonist[12], and to test the effects of a PKA inhibitor on the PKA and PKA kinase activity levels in the RPE cells and on the protective effects of the β-adrenergic agonist.

MATERIALS AND METHODS

Reagents HCQ sulphate was supplied by TCI America (Charleston, Texas, USA). Salbutamol (a adrenergic β2-receptor agonist), cell lysis buffer and protease inhibitors cocktail were supplied by Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-phospho-PKA C subunit antibody was purchased from Cell Signaling Technology (Boston, MA, USA). Rabbit anti-GAPDH antibody and anti-rabbit IgG horseradish peroxidase were purchased from Abcam (Cambridge, MA, USA). PKA inhibitor (PKA inhibitor 5-24) was obtained from TOCRIS Bioscience (Minneapolis, MN, USA). Cell culture supplies, including Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), D-Hanks and trypsin-EDTA solutions were supplied by GIBCO (Carlsbad, CA, USA). BCA protein assay kit was provided by Thermo Scientific (Rockfield, IL, USA). Image J software was obtained from NIH (Bethesda, MD, USA).

Cell Viability Evaluated by Cell Counting and Dye Exclusion Test Donor eyes were obtained from New York Eye Bank for Sight Restoration (New York, NY, USA). The Eye Bank obtained the donor's consent before the collection of the eyes. The methods for the isolation and culture of human RPE cells from the donor eyes and the study of cultured human RPE cell viability in the *in vitro* models have been reported by our group previously in detail[11-13,15-21]. Briefly, cultured human RPE cells were seeded into the 12-well plates and cultured until near confluence. HCQ was added to the medium at concentrations of 30 or 100 μmol/L. Salbutamol (10^{-5} mol/L), a β-adrenergic agonist, was added to the medium 2h before the addition of HCQ[12]. In cells treated with PKA inhibitor (PKA inhibitor 5-24), the inhibitor (10 μmol/L) was added to the medium 1h before the salbutamol. After 24h incubation, cell culture medium with floating cells were aspirated and collected. The cultures were washed by the D-Hanks solution and the washing solution was aspired and collected. Cells were

detached by trypsin-EDTA solutions at 37℃ and neutralized by FBS. Aspirated culture medium, washing solution and cell suspensions obtained by trypsin-EDTA were centrifuged. After withdrawal of the supernatant, cell pellets were resuspended in 1 mL of culture medium. Cell suspensions (50 μL) were aspirated, mixed with an equal volume of fresh prepared and filtered trypan blue solution (0.4%), and cell numbers were counted by using a hemacytometer. Viable cells (non-stained) and nonviable cells (stained blue by trypan blue) were counted separately[15].

Vacuolation Measured by Photomicrograph and Image J Aanalysis Cultured human RPE cells were incubated and treated with HCQ, salbutamol and PKA inhibitor, as described above, with the exception that the HCQ was only tested at 30 μmol/L. After 24h incubation, photomicrographs were taken with an inverted phase-contrast microscope (Olympus S70) to document morphological changes.

Ten cells were randomly selected from each group (control, HCQ, HCQ with salbutamol, and HCQ with salbutamol and PKA inhibitor). The selected cells were outlined with exclusion of the nuclei. The vacuoles were thresholded using the BW mode of the Image J software. The size of the vacuoles and cytoplasm were measured by Image J separately and expressed as the ratio of total vacuoles/cytoplasm.

Measurement of Phophos-PKA C by Western Blot Analysis RPE cells (1×10^6 cells) were plated into 25 cm^2 culture flasks, cultured with or without HCQ (50 μmol/L), salbutamol (10^{-5} mol/L) and phospho-PKA (p-PKA) C inhibitor (10 μmol/L) for 24h. Cells were harvested and micro-centrifuged. Cell pellets were collected for protein extraction. Cell lysis buffer containing protease inhibitors cocktail was used to extract protein from cells according to the manufacturer's protocol. Afterward, cells extracts were micro-centrifuged at 4℃ and the supernatants were collected. The protein levels of cells extracts were measured by Bradford protein assay. The cell extracts were separated by 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% BSA in TBST for 1h and then the primary antibodies were added. The primary antibodies included rabbit monoclonal p-PKA C antibody (1:1000 dilution), and rabbit anti-GAPDH antibody (1:10000 dilution). After incubation overnight at 4℃, secondary antibodies with anti-rabbit IgG horseradish peroxidase (1:10000) were used for the detection of specific primary antibody for 1h at room temperature. Finally, the p-PKA C expression was detected by chemiluminescence with an ECL detection kit (EMD Millipore, MA, USA). The relative photographic density was quantified by Image J software.

Measurement of PKA Kinase Activity by PKA Kinase Activity Kit The PKA kinase activity assay is based on a solid phase enzyme-linked immuno-absorbent assay (ELISA) that utilizes a specific synthetic peptide as a substrate for PKA and a polyclonal antibody that recognizes the phosphorylated form of the substrate. The levels of PKA kinase activity in the cell lysates were determined by using the PKA kinase activity kit (ENZO Life Sciences, Farmingdale, NY, USA)[14]. Briefly, RPE cells were seeded into the 12 well plates, treated and collected as described in Western blot analysis. The samples (cell lysates) and a series diluted standard activated PKA were added to the wells that were pre-coated by the specific substrate, followed by the addition of ATP to initiate the reaction. The kinase reaction was terminated and a phosphospecific substrate antibody (primary antibody) was added to the wells and bound to the phosphorylated peptide substrate. After washing, a peroxidase conjugated secondary antibody was added and bound to the primary antibody. Tetramethylbenzidine substrate was added to develop the color that is in proportion to PKA kinase activity. Stop solution was added to terminate the reaction. The intensity of the color is measured in a microplate reader at 450 nm. The standard curve was generated and linear regression analysis was performed to obtain the PKA activity.

Statistical Analysis The data was analyzed using SPSS statistical software (SPSS Inc., Chicago, IL, USA). Statistical significance of differences of the means were calculated by ANOVA one-way test in comparing data from more than two groups and Student's *t*-test in comparing data between two groups. A difference at $P<0.05$ was considered to be statistically significant. Tests were performed in triplicate. All data in text or bar graphs are shown as mean±standard deviation (SD).

RESULTS

Cell Viability The viable cells and nonviable cells in the aspirated culture medium, washing solution and cell suspensions were counted, their numbers in the four groups (control, HCQ, HCQ with salbutamol and HCQ with salbutamol and PKA inhibitor) were compared and analyzed using one-way ANOVA.

In the cultures treated with HCQ at 30 μmol/L (Figures 1 and 2), the viable cells (69% of the controls) were significantly less than that of the controls ($P<0.05$). Salbutamol significantly increased the numbers of viable cells as compared with cultures treated with HCQ alone ($P<0.05$; Figure 2). The viable cell number in HCQ with salbutamol group was slightly less than that of the controls, but the difference was statistically non-significant ($P>0.05$; Figure 2). Addition of the PKA inhibitor eliminated the protective effects of salbutamol ($P<0.05$) and the numbers of viable cells were reduced to levels of the cultures treated with HCQ alone ($P>0.05$; Figure 2). The numbers of nonviable cells in all four groups were relatively low (ranging from 0.7% to 2% of the total cells) and the differences between all groups were statistically non-significant ($P>0.05$; data not shown).
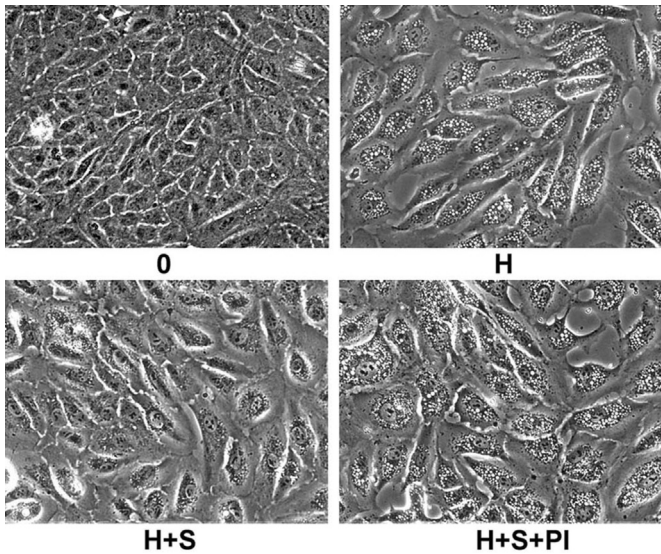
Figure 1 Phase-contrast microscopy of cultured human RPE cells treated with HCQ at 30 µmol/L, salbutamol and PKA inhibitor Cells were seeded into 12-well plates and cultured. Various factors were added into the culture medium. After 24h incubation, microphotographs were taken by phase-contrast microscopy at high magnification (×200). 0: Control, cells not treated with any of these factors; H: Treated with HCQ 30 µmol/L; H+S: Treated with HCQ and salbutamol (10^{-5} mol/L); H+S+PI: Treated with HCQ, salbutamol and PKA inhibitor (10 µmol/L). In HCQ treated cells, cells were enlarged, numerous vacuoles appeared in the cytoplasm with slightly decrease of cell numbers. Salbutamol treatment significantly decreased the number of vacuoles. PKA inhibitor increased the number of vacuoles as compared with cells treated with HCG and salbutamol only.
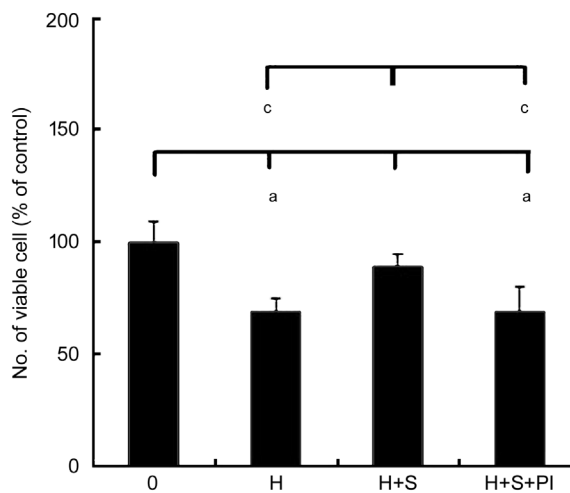

Figure 2 Effects of HCQ at 30 µmol/L, salbutamol and PKA inhibitor on cell viability of cultured human RPE cells Cells were treated as described in Figure 1. After 24h incubation, viable cells (non-stained) and nonviable cells (stained blue by trypan blue) were counted separately. Viable cells in HCQ (H) and PKA inhibitor (H+S+PI) were significantly less than that of the controls ([a]$P<0.05$). Salbutamol (H+S) significantly increased the number of viable cells ([c]$P<0.05$) and PKA inhibitor (H+S+PI) significantly decreased the viable cells ([c]$P<0.05$).

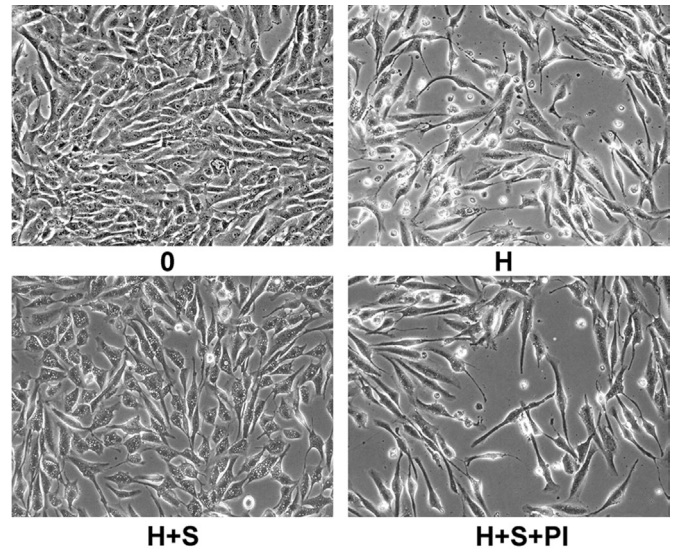**Figure 3 Phase-contrast microscopy of cultured human RPE cells treated with HCQ at 100 µmol/L, salbutamol and PKA inhibitor** Cells were seeded into 12-well plates and cultured. Various factors were added into the culture medium. After 24h incubation, microphotographs were taken by phase-contrast microscopy at low magnification (×100). 0: Control, cells not treated; H: Treated with HCQ 100 µmol/L; H+S: Treated with HCQ and salbutamol (10^{-5} mol/L); H+S+PI: Treated with HCQ, salbutamol and PKA inhibitor (10 µmol/L). In all three treated groups, cells were transformed into spindle shape, viable cells were significantly decreased and many nonviable cells floated in the medium or sill attached to the culture dish, especially in cultures treated with H and H+S+PI.

In the cultures treated with HCQ at 100 µmol/L, the epithelial morphology of RPE cells was transformed into spindle shape and many nonviable cells floated in the medium or were still attached to the culture dish (Figure 3). The number of viable cells (decreased to only 29% of the controls) was significantly less than controls ($P<0.05$; Figure 4A). The number of nonviable cells (19% of the total cells) was significantly greater than that of the controls ($P<0.05$; Figure 4B).

Salbutamol improved the viability of cells treated with HCQ, significantly increasing the numbers of viable cells and decreasing the number of nonviable cells as compared to cultures treated with HCQ alone; but both cell numbers were still significantly different than the controls ($P<0.05$; Figure 4). Addition of PKA inhibitor completely eliminated the protective effects of salbutamol ($P<0.05$), and the numbers of viable and nonviable cells were returned to levels of cultures treated with HCQ alone ($P>0.05$; Figure 4).

Vacuolation and Cell Viability Normal human RPE cells usually do not have any vacuoles, although an occasional cell may have one or two vacuoles, with a very low ratio of total vacuoles/cytoplasm size (0.2%; Figures 1, 5, and 6). Numerous vacuoles appeared in the cytoplasm in cells treated with 30 µmol/L HCQ (ranged 30-60 vacuoles per cell) and the

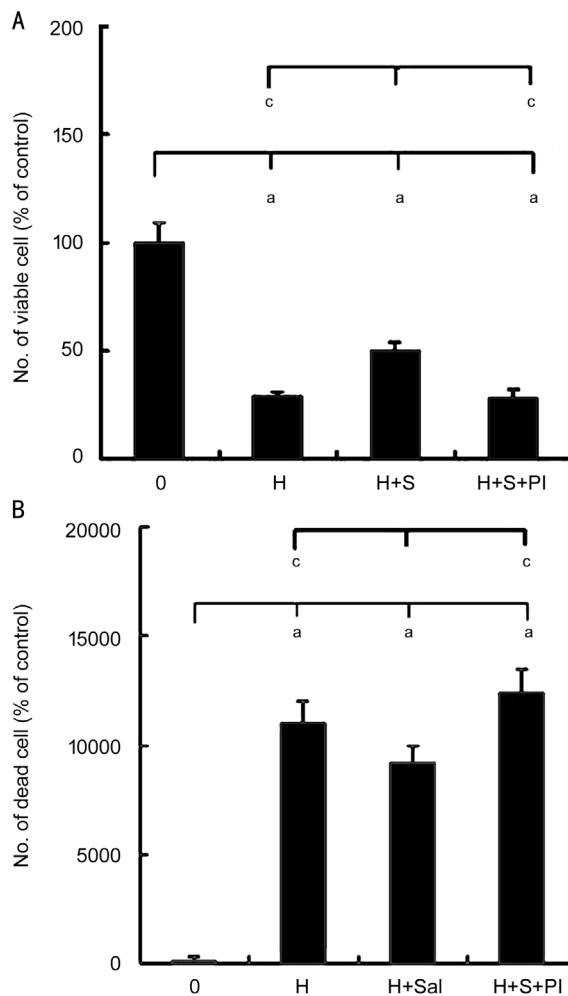**Figure 4 Effects of HCQ at 100 μmol/L, salbutamol and PKA inhibitor on cell viability of cultured human RPE cells** Cells were treated as described in Figure 3. After 24h incubation, viable cells (non-stained) and nonviable cells (stained blue by trypan blue) were counted separately. A: Viable cells in cultures treated with HCQ (H), HCQ with salbutamol (H+S) and PKA inhibitor (H+S+PI) were significantly less than that of the controls ([a]$P<0.05$). Salbutamol (H+S) significantly increased the number of viable cells ([c]$P<0.05$) and PKA inhibitor (H+S+PI) significantly decreased the viable cells ([c]$P<0.05$). B: Nonviable cells in H, H+S and H+S+PI groups were significantly greater than that of the controls ([a]$P<0.05$). Salbutamol (H+S) significantly decreased the number of nonviable cells ([c]$P<0.05$) and PKA inhibitor (H+S+PI) significantly increased the viable cells ([c]$P<0.05$).

ratio of total vacuoles/cytoplasm size (17%) was significantly greater than that of the controls ($P<0.05$; Figures 1, 5, and 6). Cells treated with HCQ and salbutamol showed a significant decrease in vacuoles within the cytoplasm (usually less than 10 vacuoles per cell), the ratio of vacuoles/cytoplasm size (2%) was significantly less than cells treated with HCQ ($P<0.05$), and not significantly different from controls ($P>0.05$; Figures 1, 5, and 6). PKA inhibitor-treated cells showed significantly greater number of vacuoles compared to cells treated with HCQ and salbutamol ($P<0.05$) alone and were not significantly
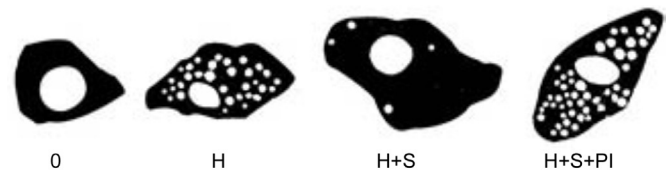

Figure 5 Cell vacuolation measured by Image J analysis Cells were treated as described in Figure 1. Microphotographs were taken by phase-contrast microscopy at high magnification (×200). Selected cells in the microphtographs were outlined with exclusion of the nuclei. The vacuoles were thresholded using the BW mode of the Image J software. Cells were black in color and the vacuoles were white in color. Examples of cells treated by Image J software. 0: Control, cells not treated with any of these factors. No vacuole could be detected. H: Cells treated with HCQ 30 μmol/L. Numerous vacuoles appeared in the cytoplasm. H+S: Cells treated with HCQ and salbutamol (10^{-5} mol/L). Very few vacuoles appeared in the cytoplasm, which were significantly less than that in the H. H+S+PI: Cells treated with HCQ, salbutamol and PKA inhibitor (10 μmol/L). Numerous vacuoles appeared in the cytoplasm.

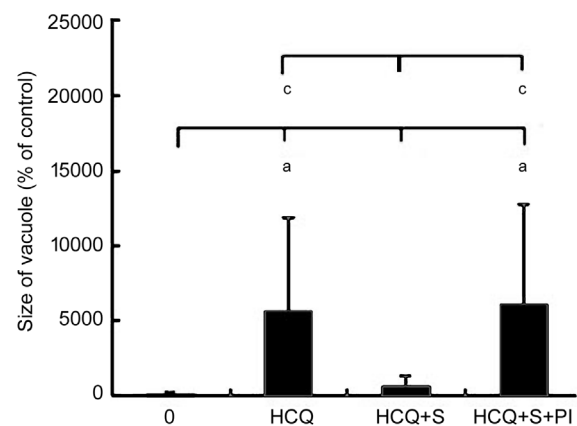**Figure 6 Effects of HCQ at 30 μmol/L, salbutamol and PKA inhibitor on cell vacuolation of cultured human RPE cells** Cells were treated as described in Figure 1. After 24h incubation, photomicrographs were taken. Ten cells were randomly selected from each group. The size of the vacuoles and cell cytoplasm were measured and compared by Image J (the ratio of total vacuoles/cytoplasm size) and expressed as the percentage of the control. HCQ at 30 μmol/L (H) and HCQ with salbutamol and PKA inhibitor (H+S+PI) significantly increased the size of vacuolation, [a]$P<0.05$. Salbutamol (H+S) significantly decreased the size of vacuolation ([c]$P<0.05$), whereas PKA inhibitor (H+S+PI) significantly increased the size of vacuolation, [c]$P<0.05$.

different from cells treated with HCQ alone ($P<0.05$; Figures 1, 5, and 6).

Western Blot Analysis Western blot analysis showed that the p-PKA C levels in the control RPE cells were reduced by the addition of HCQ (Figure 7). Sabutamol significantly upregulated the p-PKA C levels in the RPE cells as compared to cells treated with HCQ alone ($P<0.05$). PKA inhibitor
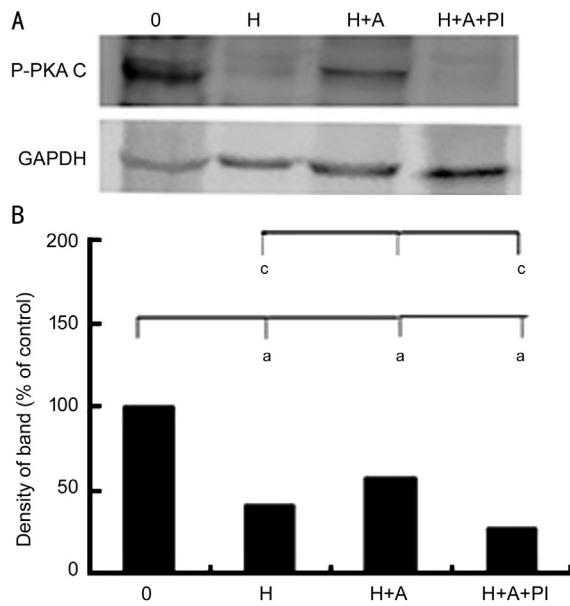
Figure 7 Western blot analysis of p-PKA C levels in RPE cells treated with HCQ, salbutamol and PKA inhibitor A: Western blot photos prepared from the cell lysates. 0: Control, cells not treated; H: Cells treated with HCQ alone; H+S: Treated with HCQ and salbutamol; H+S+PI: Treated with HCQ, salbutamol and PKA inhibitor. B: Western blot band relative density analyzed by using Image J. p-PKA levels in cell lysates treated with HCQ were significantly decreased as compared with the controls (0), [a]$P<0.05$. p-PKA levels in HCQ and salbutamol (H+S) group were significantly elevated than that of cells treated with HCQ alone (H), [c]$P<0.05$. p-PKA levels in cells treated with HCQ, salbutamol and PKA inhibitor (H+S+PI) were significantly lower than that of cells treated with HCQ and salbutamol, ([c]$P<0.05$) and than that of the cells treated with HCQ alone $P<0.05$.

(10 μmol/L) decreased the p-PKA C levels in cells treated with HCQ plus salbutamol ($P<0.05$; Figure 7). The levels of p-PKA C in cells treated with HCQ, salbutamol and PKA inhibitor were even lower than those cell cultures treated with HCQ alone ($P<0.05$; Figure 7). This suggests that this inhibitor not only eliminated the salbutamol's upregulation effects on PKA levels, but also inhibits the expression of PKA C modulated by other factors in cells treated with HCQ alone.

PKA Kinase Activity Analysis The results of PKA kinase activity analysis were parallel with the PKA analysis by Western blot analysis. PKA kinase activity in the RPE cells was inhibited significantly by HCQ and showed partially recovery by the addition of salbutamol, which was significantly greater than that of cells treated with HCQ alone, but still significantly less that that of cells not treated with HCQ (both $P<0.05$). PKA inhibitor significantly inhibited PKA kinase activity levels in cells treated with HCQ and salbutanol or cells treated with HCQ alone (both $P<0.05$; Figure 8).

DISCUSSION

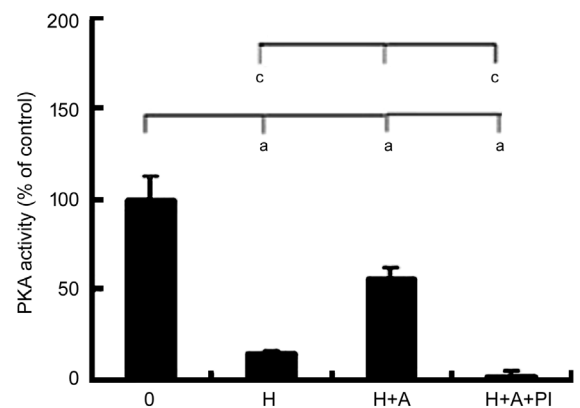Autophagy is a lysosomal degradation mechanism important

Figure 8 PKA kinase activity levels in RPE cells treated with HCQ, salbutamol and PKA inhibitor PKA kinase activity levels were measured by PKA kinase activity kits in cell lysates from four different groups. 0: Control, cells not treated; H: Cells treated with HCQ alone; H+S: Treated with HCQ and salbutamol; H+S+PI: Treated with HCQ, salbutamol and PKA inhibitor. PKA kinase activity levels in the controls (0) were significantly reduced by the addition of HCQ, [a]$P<0.05$. Salbutamol significantly increased PKA kinase activity levels as compared with HCQ, [+]$P<0.05$; but still lower than that of the control, [a]$P<0.05$. Addition of PKA inhibitor inhibited the PKA kinase activity levels in cells treated with HCQ and salbutamol, [c]$P<0.05$; and lower than that of cells treated with HCQ alone, $P<0.05$.

for cell survival under conditions of starvation, stress, or infection. To guarantee normal cellular function, cells require constitutive basal autophagic activity to keep the cytoplasm clean, prevent the accumulation of misfolded and aggregated proteins, eliminate unwanted organelles such as damaged mitochondria, and allow acquisition of basic nutrients for the building of new cellular material. Disturbance of the autophagy process leads to damage of the cell and even cell death if the disturbance is severe enough[6,22].

Lysosomal enzymes play an important role in the autophagy process and the pH of the lysosomal lumen is critical for their enzymatic activity. HCQ elevates lysosomal pH and inhibits the activity of lysosomal enzymes, ultimately blocking the autophagy process and leading to increased cell vacuolation and cell death[6-9].

We began studying the methods for isolation, cultivation and study of the functions of human RPE cells in 1980 and first developed an *in vitro* model for the studying of various functions of RPE cells (phagocytosis and retinol metabolism)[15]. We have established many human RPE cell lines from the donor eyes and used these cells for the studying of various factors modulating the growth and functions of RPE cells, to investigate the role of RPE cells in the pathogenesis of various eye diseases[15,17-21] and to explore the translation of these results to the clinical management of several retinal diseases[16]. We have developed an *in vitro* model using cultured human

RPE cells for the study of pathogenesis of HCQ retinopathy and to search the medications that may have effects for the prevention and treatment of HCQ retinopathy[11-12]. The dose- and time-dependent HCQ toxic effects on the cultured human RPE cells have been studied[11]. This model uses two different dosages of HCQ to mimic the two different stages of HCQ-induced damage of RPE cells. A moderate dosage of HCQ (30 μmol/L) that causes the vacuolation of RPE cells associated with inhibition of cell growth but does not lead to cell death. A high dosage (100 μmol/L) that causes significant death of RPE cells and reduces the viable cells to approximately one third of the untreated cells[11].

The appearance of the vacuoles begins several hours after the application of HCQ and reaches a maximal level at 24h after which vacuolation gradually decreases. Reduction in the cell viability can be clearly recognized at 24h subsequently increasing or decreasing depending upon many different factors. Therefore, in this model, the effects are best evaluated 24h after the addition of HCQ into the culture medium. These results are consistent with previous reports of dose and time effects of HCQ toxicity in RPE cells[6,23]. There are two parameters used for the evaluation these two different toxic effects, the vacuolation (the functional disturbance of lysosome with accumulation of waste causing the appearance of vacuoles) and the cell viability (the cell death). At the higher dosage that causing the cell death, the cells with many vacuoles usually die earlier, so vacuolation can only be used as a measure in the moderate dosage and cannot be used as a parameter for evaluating the effects at the large dosage.

A variety of agents have been tested by using this model. Growth factors, such as basic fibroblast growth factor, can increase the viability of cells treated with 100 μmol/L HCQ, but cannot affect the vacuolation in cells treated with 30 μmol/L HCQ, suggesting that this growth factor cannot restore the function of the lysosomes and the decrease of cell death is possibly a non-specific cell survival effect[11].

We have tested the effects of epinephrine in this HCQ toxic model and found that it decreases both the vacuolation and cell death in RPE cell treated by HCQ, indicating that the function of lysosomes can be restored and leads to the increase of cell viability. We have tested the protective effects of various adrenergic receptor agonists, which consist of α1, α2, β1, β2 and β3 receptor agonists, on RPE cells against the HCQ toxicity. Of the adrenergic receptor agonists tested, nonspecific β-receptor agonist (isoproterenol), β1-receptor agonist (prenalterol), and β2-receptor agonist (salbutamol) have potent protective effects on RPE cells; while β3-receptor agonist (CL316243) did not show significant effects. The α-adrenergic receptor agonists, clonidine (α-2) and methoxamine (α-1) did not show any protective effect. The protective effects

in these adrenergic agonists correlate well with their signal pathways, all of the effective agonists activate the cAMP signal pathway[12-13]. Therefore, the downstream of cAMP pathway (PKA pathway) was selected to investigate in the present study. Adrenergic β1 and β2 agonists bind to G protein-coupled receptors and activate the G proteins, then, adenylyl cyclase is activated, which leads to the production of cAMP. Most of the effects of cAMP are mediated by PKA. In the absence of cAMP, PKA is a stable and inactive enzyme which composed of a regulatory (R) subunit and a catalytic (C) subunit. cAMP can bind to the R subunits, lowers its affinity for the C subunit, then the C subunits can be released. The activated PKA C subunit can catalyze phosphorylation of various substrates and modulate a variety of cell functions[24-25].

The hypothesis tested in the present study is that the activation of PKA signal pathway by β-adrenergic agonist plays an important role in the protecting of RPE cells against the HCQ toxicity. To test this hypothesis, we used our *in vitro* HCQ toxicity model to evaluate the effects of HCQ, salbutamol (a β2 adrenergic receptor agonist that showed potent protective effect against the HCQ toxicity in previous studies)[12-13], and a PKA inhibitor on the cell viability, vacuolation and express of activated PKA and PKA kinase activity in human RPE cells. For the testing of this hypothesis, two important results should be evaluated by this study: 1) salbutamol should upregulate the expression of activated PKA and PKA kinase activity levels and decrease the cell vacuolation and cell death caused by HCQ; 2) PKA inhibitor should down regulate the expression of activated PKA and PKA kinase activity levels caused by salbutamol and counteract the protective effects of salbutamol on RPE cells against HCQ as evaluated by cell vacuolation and cell viability. In the present study, 1) PKA and PKA kinase activity levels were significantly elevated by salbutamol and were accompanied with the decrease in cell vacuolation in cells treated with 30 μmol/L HCQ and improvement of cell viability in cells treated with 100 μmol/L HCQ; 2) PKA inhibitor reversed salbutamol-induced elevation of PKA and PKA kinase activity levels and counteracted the protective effects of salbutamol against the HCQ toxicity. Cell vacuolation was increased and cell viability was decreased significantly due to the addition of PKA inhibitor and both parameters were reversed to the levels of cultures treated with HCQ alone.

With this study, all of the requirements for the testing of our hypothesis have been satisfied, validating the hypothesis that PKA pathway plays an important role in the protective effects of β2-adrenergic agonist on the RPE cells against HCQ toxicity.

Many β1 and β2 adrenergic agonists have been developed in recent decades and have been safely applied clinically for long-term management of various diseases with limited side-effects[26]. Perhaps, off-label repurposing of one of these

medications may serve as a promising agent for the prevention and treatment of HCQ retinopathy.

The limitation of this study was that the effects of β2 adrenergic agonist on the PKA signal pathway and the protection of RPE cells against the HCQ toxicity were only tested in cultured cells. Therefore, it requires careful evaluated by experimental animal studies prior to the clinical trial.

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