Protection of photoreceptors by intravitreal injection of the Y-27632 Rho-associated protein kinase inhibitor in Royal College of Surgeons rats

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Abstract. Retinitis pigmentosa (RP) is an inherited retinal disease, which is characteristic by degeneration of the rod and cone photoreceptors. The present study aimed to assess the protective effects on photoreceptors of intravitreal injection of Y-27632, a specific inhibitor of Rho-associated protein kinase (ROCK), in a Royal College of Surgeons (RCS) rat model. Different concentrations of Y-27632 (1-50 mM) were administered by intravitreal injection into the RCS rats. The effects of Y-27632 were recorded using electroretinography (ERG), measuring the thicknesses of the retinal outer nuclear layer (ONL) and examination of apoptotic markers using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and western blot analysis. Treatment of the eyes with Y27632 at 10 or 50 mM, led to a 30% increase in a- and b-wave amplitudes in ERG, and an increase in ONL thickness by 10%, compared with the 1 mM Y-27632-treated and vehicle (phosphate-buffered saline; PBS)-treated groups. In addition, eyes treated with 10 mM Y27632 exhibited a 90% decrease in TUNEL-positive cells, accompanied by decreased protein expression levels of active caspase 3 and Bax by 50%, and a 90% increase in the ratio of Bcl-2/Bax, compared with the PBS-treated groups. These data suggested that Y-27632 protected retinal function by inhibiting the apoptosis of photoreceptor cells in the RCS rat model. The present study demonstrated for the first time, to the best of our knowledge, to report the use of Y-27632 for protection against RP in an RCS rat model. Y-27632 may be a potential candidate for the treatment of human RP.

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Introduction

Retinitis pigmentosa (RP) describes a group of inherited retinal diseases, characterized by the progressive loss of photoreceptors, resulting in night blindness and visual field constriction (1). The worldwide prevalence of RP is ~1/4,000, which affects >1 million individuals. The family of disease that are phenotypically recognized as RP consists of >150 genotypically distinct entities (2). Despite the different genotypes, the loss of vision in RP is associated with the death of photoreceptor cells. Previous studies have indicated that apoptosis is the dominant mechanism of photoreceptor death in Royal College of Surgeons (RCS) rats, which are one of the animal models of RP (3,4). Portera-Cailliau *et al* (5) suggested that retinal degeneration may be slowed by interfering with apoptotic mechanisms. Therefore, therapeutic strategies, which target a common mechanism of photoreceptor death among a multitude of genotypes may offer a broad clinical application for the treatment of RP.

It has been well-documented that the small GTP-binding protein, Rho, contributes to the regulation of neurite outgrowth (6-8) and apoptosis (9) in neuronal cells. In addition, Rho-associated protein kinase (ROCK), one of the key effectors of Rho, is involved in growth factor signaling (10,11), the regulation of apoptosis (12) and cell survival (13). These proteins are abundantly expressed in various types of cell (14), including those in the retina (15-17). Previous reports have demonstrated that the activation of RhoA, one of the Rho family GTPases, can induce retinal degeneration (18). The intravitreal administration of HA-1077 or Y-27632, which are specific ROCK inhibitors, has been observed to improve retinal function in a mouse model of Huntington's Disease with progressive retinopathy (19), and to rescue retinal ganglion cells in rat models of diabetes and transient retinal ischemia (18). The present study hypothesized that the photoreceptor degeneration in RP may be treated by intravitreal administration of Y-27632, via the inhibition of apoptosis in RCS rats. This is the first time, to the best of our knowledge, that Y-27632 that has been used for RP in RCS rat models.

Materials and methods

Animal model. A total of 20 male and 20 female dystrophic RCS rats (rdy+p+; 21 days-old), obtained from the Third Military Medical University (Xian, China) were used in the present study. The rats were housed in cages (one rat/cage), maintained in standard conditions (room temperature 23±2C; relative humidity 60±10%; 12 h light/dark cycle), and were fed a standard laboratory diet with *ad libitum* access to water. Prior to all experiments, the ocular surface integrity was examined by slit-lamp microscope (OMS-85; Topcon, Tokyo, Japan). At the point of intravitreal injection, development of the rods and cones in the rats had been completed (20) and electroretinograph (ERG) responses were normal amplitudes (21). The rats were anesthetized and sacrificed by intraperitoneal injection of 10% chloral hydrate (China Otsuka Pharmaceutical Co., Ltd., Tianjin, China), following which the eyes were immediately enucleated. The animals were maintained and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the present study was approved by the animal ethics committee of Zhongshan Ophthalmic Center, Sun Yat-sen University (Guanzhou, China; approval no. ID:2012-12).

Experimental design. A total of 40 RCS rats were used for ERG recording and measurements of the ONL thickness. The rats were divided into four groups (n=10 in each group): 1 mM Y27632 intravitreal injection, 10 mM Y27632; 50 mM Y27632 and phosphate-buffered saline (PBS) control groups. A total of 20 RCS rats were used for terminal deoxynucleotidyl transferase-mediated biotinylated-dUTP nick-end labeling (TUNEL) assays and to determine the expression levels of active caspase 3, Bax and Bcl-2. These rats were divided into two groups (n=10 in each group): A 10 mM Y27632 and a PBS (1 μl) control group.

Y-27362 administration. The dosage effects of Y-27632 (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) on the morphology and function of the retina were assessed by injecting 1, 10 or 50 mM Y-27632 into the vitreous (0.1, 1 and 5 mM final concentrations, respectively, on day 21 (n=10). The doses were selected based on the successful neuroprotection of retinal ganglion cells, demonstrated using a dose of 100 nmol/eye, which was equal to the final concentration of 1 mM used in the present study (18,22). The inhibitory effects of Y-27632 on the apoptosis of the photoreceptor were analyzed by injecting 10 mMY-27632 on day 21 (n=10) (20). Each concentration of Y-27632 was diluted in PBS to the same final volume (1 μl) for the intravitreal injection. The control group was intravitreally injected with 1 μl PBS, instead of Y-27632 (n=10).

Intravitreal injections were performed under an operating microscope (OPMI VISU 200; Carl Zeiss AG, Oberchoken, Germany) , using a microsyringe (Hamilton, Reno, NV, USA) with a 30-gauge needle (Hamilton). The needle was inserted ~1 mm behind the corneal limbus.

ERG recording. The ERG recording was performed on day 28, according to Mandal *et al* (23). ERGs were monitored using a Neuropack α electophysiological recorder (Nihon Kohden Corp., Tokyo, Japan). Briefly, following 12 h dark adaptation under dim red light, the animals were anesthetized by intramuscular administration of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine (Shenyang Sinqi Pharmaceutical Co., Ltd., Shenyang, China). The pupils were dilated with 0.5% tropicamide (Shenyang Sinqi Pharmaceutical Co., Ltd.) and body temperature was maintained at ~37˚C with a heating pad (Beijing Gaoshiyuanwang Technologies Co., Ltd., Beijing, China). ERGs were recorded from each eye using a gold loop electrode (Beijing Gaoshiyuanwang Technologies Co., Ltd.) on the corneal surface, maintained with Lacrigel lubricating ointment (Laboratoires Europhta, Monaco). A reference electrode was attached to the shaven skin on the head and a ground electrode was clipped to the animal skin of the tail. The amplitudes of a-wave and b-wave were measured from the average of three responses by a set of three flashes of stimulation, using two flash intensities, 1,000 and 2,000 cd·s/m^2.

Measurement of the ONL thickness. Following the ERG recording, the animals were sacrificed by carbon dioxide asphyxiation. The animal's eyes were removed, fixed with 4% paraformaldehyde and embedded in paraffin. Sections (5 μm thick) of eyeballs were cut along the vertical meridian through the optic nerve head. The sections were dewaxed with xylene, rehydrated with anhydrous ethanol and stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA). The ONL thickness was measured under a light microscope (Axioplan2; Carl Zeiss Meditec, Inc., Jena, Germany). Measurements were made at 300 μm intervals in nine defined areas, starting at the optic nerve, and extending toward the superior and inferior ora serrata, according to LaVail *et al* (24). The ONL thickness measurements were averaged within each group to provide a single value for statistical analysis.

TUNEL assay. A TUNEL assay was performed to detect apoptosis on the cryosections of the rat eyes using the *in situ* Cell Death Detection TMR Red kit (Roche Diagnostics, Indianapolis, IN, USA). For staining of the sections, the samples were permeabilized for 2 min in cold PBS, containing 0.1% Triton X-100 and were subsequently incubated at 37˚C in the TUNEL reaction mix, which contained nucleotides and terminal deoxynucleotidyl transferase (TdT). Incubation in the absence of the TdT enzyme was performed as a negative vehicle control. The sections were subsequently mounted (VECTASHIELD mounting medium with DAPI; Vector Laboratories, Inc., Burlingame, CA, USA), and visualized by confocal microscopy (LSM510; Carl Zeiss AG). The apoptotic index of the ONL was calculated as the number of TUNEL-positive nuclei / number of photoreceptor cell nuclei, as previously described by Kyger *et al* (25).

Western blot analysis. The rat retinas were dissected using a surgical microscope and lysed in buffer, containing 1 M Tris-HCl (pH 7.5), 1% Triton X-100, 1% nonidet P-40, 10% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.5 M EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The retinal protein concentrations were determined using a bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL, USA). The retinal proteins (25 μg) were separated using SDS-PAGE and transferred onto a

polyvinylidine difluoride filter membrane (Millipore, Bedford, MA, USA). The membranes were then blocked with skimmed milk at room temperature for 2 h. Subsequently, the membrane was incubated with primary polyclonal antibodies against active caspase 3 (anti-mouse; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Bcl-2 (anti-mouse; 1:1,000; Sigma-Aldrich), Bax (anti-rabbit; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) or β-actin (anti-mouse, 1:1,000; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. The membrane was subsequently incubated with goat-anti-rabbit or goat-anti-mouse conjugated horseradish peroxidase secondary antibody (1:2,000; Southern-Biotech, Birmingham, AL, USA) for 2 h at room temperature. The membranes were visualized using an enhanced chemiluminescence system (Pierce Biotechnology, Inc.).

Statistical analyses. One-way analysis of variance, followed by Bonferroni's multiple-comparison test was used for statistical analyses. The data are expressed as the mean ± standard error of the mean. Statistical analyses were conducted using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Protective effect of Y-27632 on the retinal function of RCS rats. To investigate the effect on the retinal function of intravitreal injection of Y-27632, the present study analyzed the scotopic mixed rod-cone ERG a- and b-wave amplitudes 1 week following injection. Treatment with 10 or 50 mM Y-27632 significantly increased the a- and b-wave amplitudes by ~30%, compared with the 1 mM Y-27632-treated and control groups ($P<0.01$). However, no significant changes were observed between the 1 mM Y-27632-treated and control groups ($P>0.05$), or between the 10 mM and 50 mM Y-27632-treated groups ($P>0.05$; Fig. 1A-C).

Protective effect of Y-27632 on the ONL thickness of RCS rats. The present study then investigated whether retinal morphology was altered following Y-27632 injection by analyzing the ONL thickness. No significant differences were observed in the ONL thickness between the control and 1 mM Y-27632-treated eyes, however, the ONL thicknesses in the 10 and 50 mM Y-27632-treated eyes were significantly higher than those in the control and 1 mM Y-27632-treated eyes 1 week post-injection. No significant differences were observed between the ONL thicknesses of the eyes in the 10 and 50 mM Y-27632 groups (Fig. 2A and B). According to these findings, the dosage of 10 mM Y-27632 was used as the minimum and effective concentration in the subsequent experiments.

Protective effect of Y-27632 on photoreceptor apoptosis in RCS rats. To determine whether Y-27632 affected the apoptosis of photoreceptors in RCS rats, *in situ* labeling using a TUNEL assay was performed in the retinal tissues of the rats. An abundance of TUNEL-positive cells (red) were detected in the retina of the control group 1 week post-injection. However, the number of TUNEL-positive cells in the 10 mM Y-27632-treated eyes was markedly lower. In each group, the TUNEL-positive

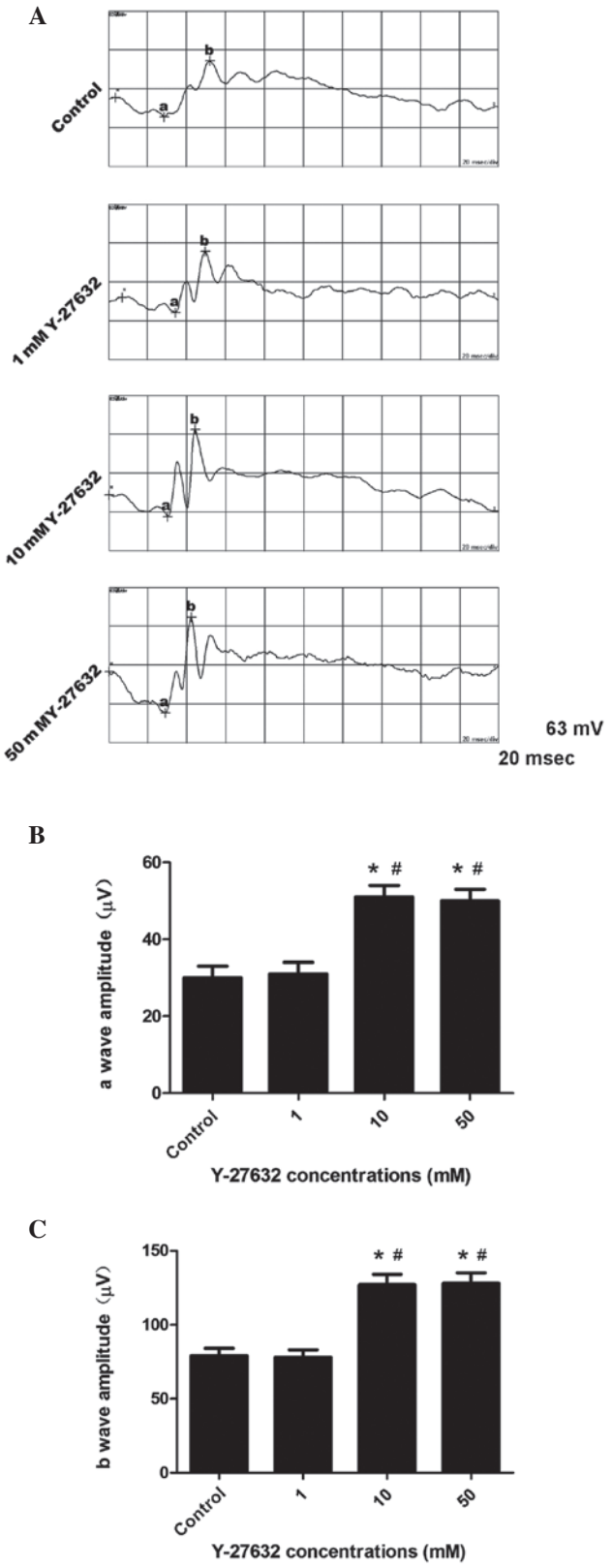

Figure 1. Treatment with Y-27632 (10 or 50 mM) inhibits the decrease of a- and b-wave amplitude in RCS rats. (A) ERG recording, (B) a-wave amplitude and (C) b-wave amplitude 1 week post-injection. No significant changes in the a- and b-wave amplitude were observed in the Y-27632-treated (1 mM) eyes compared with the controls ($P>0.05$; n=10). Y-27632-treated (10 or 50 mM) eyes exhibited 30% higher a- and b-wave amplitudes compared with the 1 mM Y-27632-treated eyes and the controls (n=10; $^{*}P<0.01$, compared with the 1 mM Y-27362-treated eyes and $^{\#}P<0.01$, compared with the controls). However, no significant changes were observed between the eyes treated with 10 and 50 mM Y-27362 ($P>0.05$; n=10). RCS, Royal College of Surgeons; ERG, electroretinography.

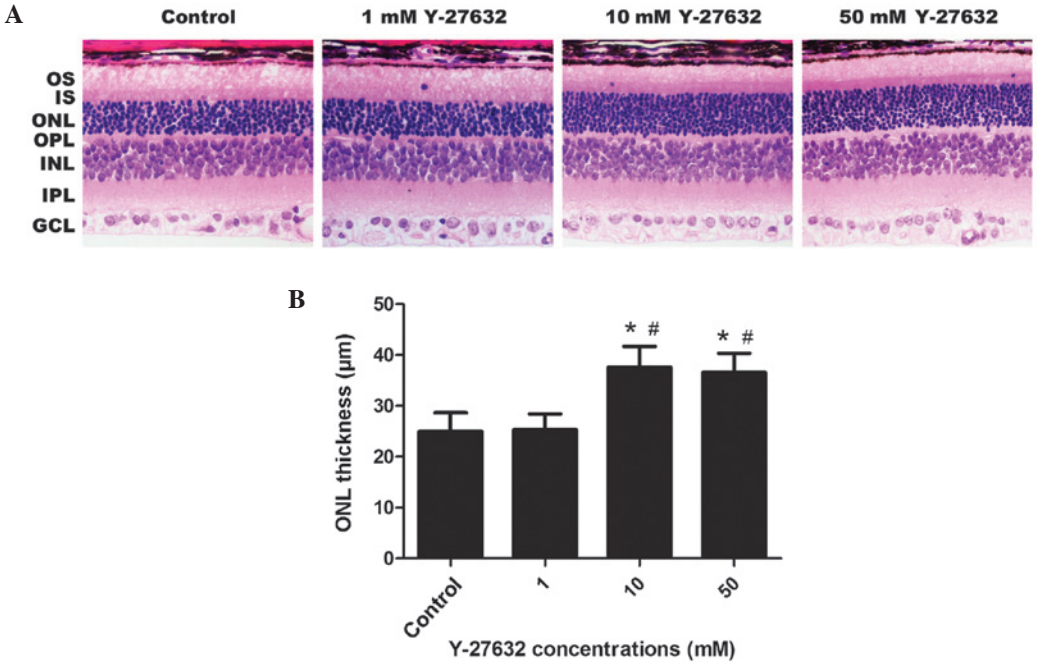

Figure 2. Treatment with Y-27632 (10 or 50 mM) alteres retinal morphology in RCS rats. (A) Representative retinal sections are shown. (B) Quantification of the ONL thickness 1 week post-injection. Y-27632-treated (1 mM) eyes demonstrated no significant changes in ONL thickness compared with the controls (n=10; P>0.05). Y-27632-treated (10 or 50 mM) eyes exhibited 25% thicker ONLs compared with the 1 mM Y-27632-treated eyes (*P<0.01; n=10) and controls ($^{\#}$P<0.01). n=10). However, no significant changes was observed between the eyes treated with 10 mM and 50 mM Y-27362 (P>0.05; n=10). Scale bar=40 μm). RCS, Royal College of Surgeons; OS, outer surface, IS, inner surface; ONL, outer nuclear layer, INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

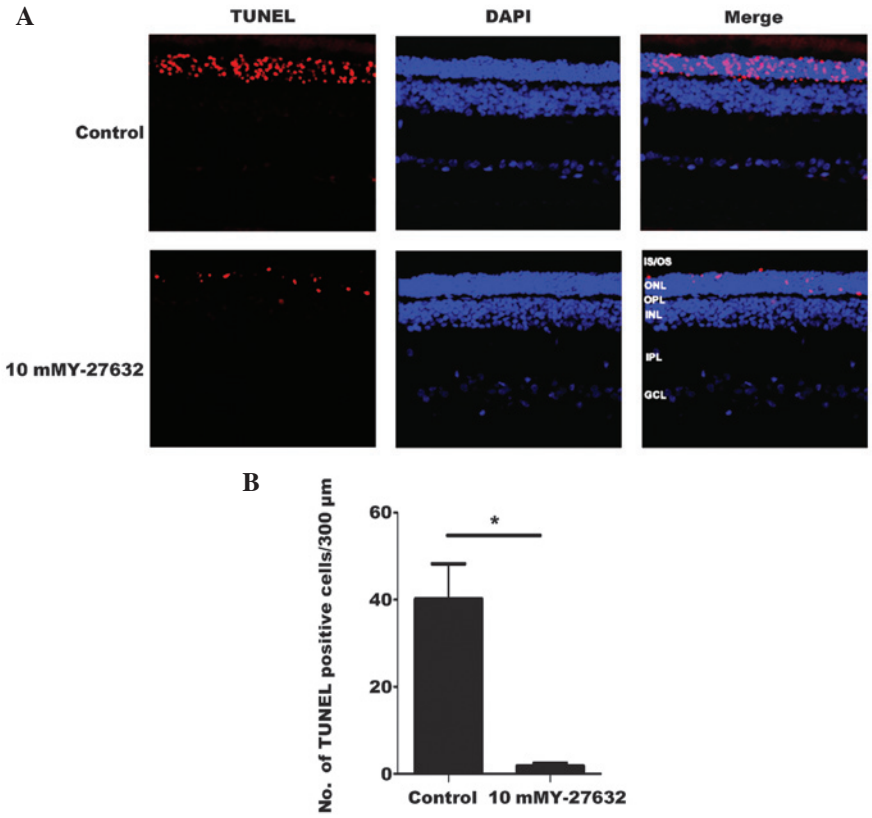

Figure 3. Analysis was performed using a TUNEL assay and visualization under confocal microscopy. The Y-27632 inhibited the apoptosis of photoreceptors induced in the RCS rats. (A) Abundant levels of TUNEL-positive cells (red) were observed in the ONL of the control retinas 1 week post-injection, however, a marked reduction in TUNEL-positive cells (red) was detected in the 10 mM Y-27632-treated retinas. Representative microscopy images are shown (scale bar=40 μm). (B) Administration of Y-27632 significantly decreased the levels of apoptosis 1 week post-injection compared with the control (*P<0.01; n=10). The nuclei were counterstained with DAPI (blue). RCS, Royal College of Surgeons; ONL, outer nuclear layers; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4',6-diamidino-2-phenylindole;OS, outer surface, IS, inner surface; ONL, outer nuclear layer, INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

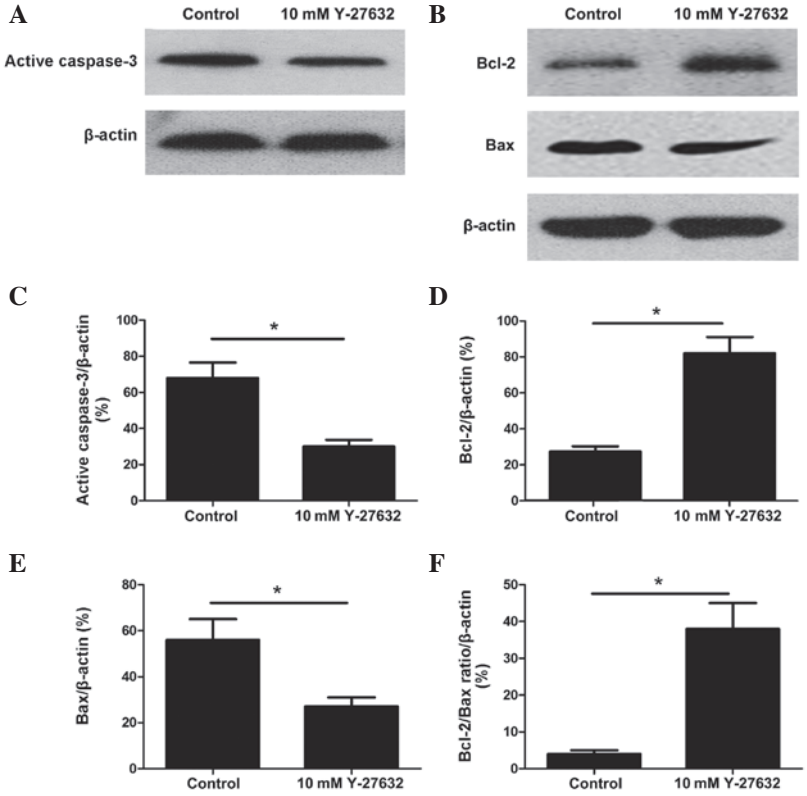

Figure 4. Effects of treatment with Y-27632 on the expression levels of active caspase 3, Bcl-2 and Bax in the retina. The protein expression levels of active (A) caspase 3, (B) Bcl-2 and Bax in the retina were determined using western blot analysis. (C) Protein expression levels of active caspase 3 were significantly lower in the 10 mM Y-27632-treated rats compared with the normal saline (NS)-treated rats *P<0.01; n=10). The protein expression levels of (D) Bcl-2 and (E) Bax in the retinas were determined using western blot analysis and the (F) Bcl-2/Bax ratio was calculated. Treatment with 10 mM Y-27632 caused a significant increase in the Bcl-2/Bax ratio compared with the NS-treated rats at day 28 (*P<0.01; n=10).

cells were present only in the ONL. The administration of 10 mM Y-27632 significantly decreased the apoptosis of photoreceptors compared with the PBS-injected control group (P<0.01), as shown in Fig. 3A and B).

Y-27632 increases the protein expression of Bcl-2 and decreases the protein expression levels of caspase 3 and Bax in RCS rats. The present study investigated the effect of Y-27632 on the expression levels of caspase 3, Bax and Bcl-2 in the RCS rat retina via western blot analysis. The protein expression levels of active caspase 3 and Bax in the 10 mM Y-27632-treated retinas markedly decreased 1 week post-injection. However, administration of 10 mM Y-27632 markedly increased the protein expression of Bcl-2. Furthermore the Bcl-2/Bax ratio in the 10 mM Y-27632-treated retinas was 10-fold higher than that in the PBS-treated retinas (Fig. 4A and B). The difference in levels of active caspase 3, Bax and Bcl-2, and the Bcl-2/Bax ratio were statistically significant between the 10 mM Y-27632-treated and PBS-treated groups (P<0.01; Fig. 4C-F).

Discussion

Previous studies have demonstrated that RhoA is sufficient to induce the neuronal excitotoxic pathway, which indicates that Rho is an essential component of the excitotoxic neuronal cell death pathway (26). It is understood that treatment with Y-27632 inhibits neurodegeneration in models of huntingtin degradation (27,28), Parkinson's disease (29) and Alzheimer's disease (30). In addition, a previous study demonstrated that HA-1077, or Fasudil, which is another selective inhibitor of ROCK, is an efficacious therapeutic molecule that slows the progression of photoreceptor degeneration (19). Furthermore, it has been reported that Y26732-supplemented culture media reduces dissociation-induced apoptosis *in vivo* and *in vitro*. Koyanagi *et al* (31) revealed that Y26732 rescues transplanted neural precursor cells from apoptosis in a mouse transplantation model. Claassen *et al* (32) also demonstrated that supplementation of cell culture media with 10 μM Y-26732 efficiently reduced apoptosis and increased the survival and proliferation of dissociated human embryonic stem cell-derived neural progenitor cells *in vitro*. Collectively, these findings suggested that Y-26732 can be used for the treatment of degeneration of photoreceptors in RP.

The present study demonstrated that intravitreal injection of the Y-27632 ROCK inhibitor prevented photoreceptor degeneration in RCS rats. Human RP can be diagnosed by changes in ERG. In the scotopic flash ERG, the a-wave primarily represents the mixed function of cones and rods, and the b-wave predominantly reflects light-induced depolarization of ONL bipolar cells, but may be altered by the activity of other cells, including the responses of Müller cells (33). The present study demonstrated significant preservation of a- and b-wave amplitudes in the ERG assays in the retinal tissues of rats treated with 10 and 50 mM Y-27632 1 week post-injection. In addition, the numbers of cells in the ONL in the 10 and 50 mM Y-27632-treated groups were significantly

higher, compared with those in the 1 mM-treated and the vehicle-injected eyes. The present study also demonstrated that no preservation of photoreceptors occurred in the 1 mM Y-27632-treated group, whereas 10 and 50 mM Y-27632 did preserve the photoreceptors. These findings indicated that, for the preservation of photoreceptors in RCS rats, 10 or 50 mM Y-27632 provided an effective dose. Therefore, a minimum and effective concentration of 10 mM Y-27632, was used in the subsequent experiments.

These findings were supported by the effect on the apoptosis of photoreceptors. Apoptosis is the predominant mechanism of photoreceptor degeneration in RCS rats (4). As described previously in RCS rats (34), the photoreceptor outer segments are irregularly aligned, with vacuolation in the inner segments and TUNEL-positive cells in 50% of the outer retinal layers on day 30 (4). The observations in the present study using PBS-injected RCS rats were consistent with these findings. By contrast, when 10 mM Y-27632 was administered intravitreally, the number of TUNEL-positive cells (red) in the outer retina was significantly reduced.

A number of studies have suggested that activation of the caspase family is important in the execution of apoptotic events (35,36). The activation of caspase-like proteases is important in the apoptosis of photoreceptors in RCS rats (37). In the present study, western blot analysis revealed that the expression of active caspase 3 in the retina of the RCS rats was decreased following treatment with 10 mM Y-27632. The Bcl-2 family of proteins, consisting of pro-apoptotic Bax and anti-apoptotic Bcl-2 members, is important in the regulation of neuronal apoptosis. Apoptosis can be inhibited by the inhibition of the expression of the apoptosis-associated genes, including Bax, or by the overexpression of Bcl-2 (38,39). Previous studies have demonstrated that Bax is upregulated in photoreceptors and Bcl-2 was not downregulated in RCS rat retinas on day 28 compared with day 21 (40). The role of the Bcl-2 family in the apoptotic process of photoreceptors remains to be elucidated (41). The present study demonstrated that the protein expression of Bcl-2 and the ratio of Bcl-2/Bax were increased, while the protein expression of Bax was reduced in the 10 mM Y-27632-treated retinas compared with the PBS-treated retinas on day 28. Taken together, these findings indicated that Y-27632 may inhibit the apoptosis of photoreceptors via a caspase-dependent pathway in the RCS rat, and the Bcl-2 family may be involved in this process.

In conclusion, the present study demonstrated that a single intravitreal injection of Y-27632 had a significant protective effect on photoreceptor degeneration, morphologically and functionally, by inhibiting the apoptosis of photoreceptors in the RCS rat retinas. Therefore, the intravitreal administration of Y-27632 is a potential candidate as a novel therapeutic strategy for photoreceptor degeneration, including RP. However, further investigation of the underlying mechanisms of Y-27632 on photoreceptor protection is required to improve the efficiency of protection for clinical therapeutic use.

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