



Full Paper

Carteolol hydrochloride reduces visible light-induced retinal damage *in vivo* and BSO/glutamate-induced oxidative stress *in vitro*



Masato Matsuo ^{a,b}, Yoshiki Kuse ^b, Kei Takahashi ^b, Keiichi Kuwahara ^c, Masaki Tanito ^a, Sachiko Kaidzu ^a, Masamitsu Shimazawa ^b, Hideaki Hara ^b, Akihiro Ohira ^{a,*}

^a Department of Ophthalmology, Shimane University Faculty of Medicine, Izumo, Japan

^b Molecular Pharmacology, Department of Biofunctional Evaluation, Gifu Pharmaceutical University, Gifu, Japan

^c Division of Dermatologicals and Ophthalmologicals, Otsuka Pharmaceutical Co., Ltd., Osaka, Japan

ARTICLE INFO

Article history:

Received 13 September 2018

Received in revised form

3 November 2018

Accepted 26 November 2018

Available online 6 December 2018

Keywords:

Carteolol hydrochloride

Light-induced retinal damage

Oxidative stress

Antioxidative potential

Reactive oxygen species

ABSTRACT

The purpose of this study was to determine whether carteolol eye drops, a β -adrenoceptor antagonist used as an intraocular hypotensive agent, has protective effects against the light-induced oxidative stress in retina. Dark-adapted pigmented rats were pre-treated with topical carteolol ophthalmic solution or saline and then exposed to visible light. The effects on electroretinogram (ERG), morphology, oxidative stress, and expression of mRNAs in the retinas were determined. The L-buthionine-(S,R)-sulfoximine (BSO)/glutamate-induced oxidative stress in 661 W cells, a murine photoreceptor cell line, was evaluated by cell death assays, production of reactive oxygen species (ROS), and activation of caspase. *In vivo* studies showed that exposure to light caused a decrease in the amplitudes of ERGs and the outer nuclear layer (ONL) thickness and an increase of the 8-hydroxy-2'-deoxyguanosine (8-OHdG)-positive cells in the ONL. These changes were significantly reduced by pre-treatment with carteolol. Carteolol also significantly up-regulated the mRNA levels of thioredoxin 1 and glutathione peroxidase 1 compared to saline-treated group. Moreover, carteolol and timolol, another β -adrenoceptor antagonist, significantly inhibited BSO/glutamate-induced cell death and reduced caspase-3/7 activity and ROS production *in vitro*. Therefore, carteolol could protect retina from light-induced damage with multiple effects such as enhancing the antioxidative potential and decreasing the intracellular ROS production.

© 2018 The Authors. Production and hosting by Elsevier B.V. on behalf of Japanese Pharmacological Society. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

The harmful effects of excessive visible white light exposure on the mammalian retina, which is one of the factors of age-related macular degeneration (AMD) occlusion and progression have been demonstrated by various studies.^{1–3} Light-induced retinal damage is considered to be due to photo-oxidative stress,² and reactive oxygen species (ROS) are produced by light exposure in the retina. ROS evoke photoreceptor degeneration,³ which can be prevented, or slowed, by various antioxidants.⁴

Carteolol hydrochloride (carteolol; 5-(3-tert-butylamino-2-hydroxy) propoxy-3, 4-dihydrocarbostyryl hydrochloride) has

non-selective β -adrenoceptor inhibitory activity and is commonly used as an intraocular hypotensive agent.⁵ We showed earlier that carteolol possessed hydroxyl radical (\bullet OH) scavenging ability, which can protect the cornea from UV-induced oxidative stress,⁶ and also had the ability to scavenge the superoxide anion radical (O_2^-).⁷ Fujio et al showed the accumulation and tendency of saturation of carteolol in the retina and choroid with instillation of ¹⁴C-carteolol eye drops.⁸ Topical carteolol should reach retina and choroid through the cornea and the sclera followed by diffusion, and through the conjunctiva via the posterior ocular circulation like other topical beta-blockers, which have chemical structures similar to that of carteolol.⁹ These results indicated that carteolol may also protect the retina from photo-oxidative stress. Thus, the purpose of this study was to evaluate the ability of carteolol to protect the retina physiologically and morphologically against excessive visible light, and to determine the cytoprotective effects of carteolol using 661 W cells, a murine photoreceptor cell line.

* Corresponding author. Department of Ophthalmology, Shimane University Faculty of Medicine, Enya 89-1, Izumo, Shimane, 693-8501, Japan. Fax: +81 853 20 2278.

E-mail addresses: mmpeaceful@yahoo.ne.jp, matsuondmc@gmail.com (A. Ohira).

Peer review under responsibility of Japanese Pharmacological Society.

2. Materials and methods

2.1. Test samples

For the *in vivo* studies, 2% carteolol hydrochloride ophthalmic solution (Mikelan[®] ophthalmic solution 2%) and saline were purchased from the Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). For the *in vitro* study, carteolol hydrochloride was obtained from Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd (Tokyo, Japan), and timolol maleate (timolol) was obtained from LKT Laboratories (St. Paul, MN, U.S.A.). The test solutions were prepared at the time of use by dissolving in Ca, Mg-free phosphate-buffered saline [PBS (-)].

2.2. Animals

Six-week-old, male Brown-Norway pigmented rats (Charles River Japan, Inc., Yokohama, Japan) were used. Before the start of the experiment, they were housed in a dark room (under 5 lux) at a temperature of 20–24 °C and humidity of 45–65% for more than a week. All animals were handled in accordance with the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research, and all experiments were approved and monitored by the Institutional Animal Care and Use Committee of Shimane University.

2.3. Retinal damage induced by visible light irradiation

A 2% carteolol solution or saline was applied topically to both eyes (10 µL/eye) of the rats twice/day for 2 weeks in the dark room. Prior to the light exposure, the pupils were dilated with topical tropicamide and phenylephrine hydrochloride (Midrin[®]-P ophthalmic solution; Santen Pharmaceutical Co., Ltd., Tokyo, Japan). One hour after the last instillation of the carteolol or saline, the unanesthetized rats were exposed to white fluorescent light (Toshiba FLR20S-W/M; Toshiba Co. Ltd, Tokyo, Japan) at 8000 lux for 1 h to evaluate the effects of the light on the Electroretinogram (ERG) and on the thickness of the outer nuclear layer (ONL), and for 3 h to evaluate the formation of 8-OHdG in the retinal tissue. After the irradiation, the rats were returned to the standard housing conditions.

2.4. Electroretinogram

At 4 days after the light irradiation, the rats were anesthetized with intramuscular injection of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) and prepared for the ERG recordings by dilating the pupils of both eyes with Midrin[®]-P. The ERGs were picked up with a bipolar contact lens electrode with an embedded light emitting diode (LE-1000, Mayo Corp., Inazawa, Japan). The reference electrode was placed on the ear and a ground electrode placed on the head. The ERGs were elicited by a stimulus intensity of 2×10^4 cd/m² with a duration of 10 ms.

2.5. Thickness of ONL

After the ERG recordings, the rats were euthanized by exsanguinations and both eyes were enucleated. The eyes were immersed in a fixative solution containing 3% glutaraldehyde-6% formaldehyde. The eyecups were embedded in paraffin, and 5 µm sections were cut through the optic nerve head. The sections were stained with hematoxylin and eosin (H&E), and the thickness of the ONL and the total retinal thickness were measured at 500 µm superior to the optic nerve head. Because oxidative stress is known to induce severe atrophy of the inner layer of the retina,³ the thickness of the ONL was expressed as the ratio to the total retinal thickness

to eliminate any influence caused by changes in the thickness of the whole retina.

2.6. 8-OHdG-positive cells in ONL

Paraffin-embedded sections of the ocular tissues were prepared by the standard method (thickness, 5 µm). The sections were immunostained with a mouse monoclonal antibody against 8-hydroxy-2'-deoxyguanosine (8-OHdG; NOF Corp., Tokyo, Japan). The immunohistochemical staining was performed by rinsing the deparaffinized sections in 0.01 M of PBS for 30 min and then pre-incubated with 10% normal goat serum in 0.01 M PBS for 1 h. The sections were then incubated with the monoclonal antibody of 8-OHdG diluted by 1:20 in a solution of 10% goat serum in 0.01 M PBS containing 0.3% (v/v) Triton X-100 overnight at 4 °C. The immunoreactivity was made visible by the ABC Method (Vectastain Elite ABC kit, Vector Lab., Burlingame, CA, USA) with color development using 3,3'-diaminobenzidine (DAB). One section from each eye was photographed and assessed. Our previous report showed that retinal damage induced by light exposure was more severe in superior retina, and thinning of ONL was observed from 0.5 to 3.5 mm superior to the optic nerve head.¹⁰ Therefore, the numbers of photoreceptor cells and 8-OHdG-positive photoreceptor cells were counted with visual observation in a 200 µm field of 500–700 µm superior to the optic nerve head. From these cell counts, the percentage of 8-OHdG-positive cells in the ONL was calculated.

2.7. Measurements of mRNA expression of antioxidant enzymes in retinal tissue

The retinas of dark-adapted rats after instillation of either 2% carteolol or saline for 2 weeks were studied. The both retinas from each rat were combined and the total RNA was extracted by Trizol (Invitrogen, Life Technologies Inc., Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized from 2 µg of total RNA with oligo (dT)₂₀ using a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. The resulting cDNA was amplified by PCR using a Platinum Taq DNA polymerase mix (Invitrogen). The primers used for the amplification were:

thioredoxin-1 (TRX1),
forward, 5'-TCTGCCGAACTCGTGTGG-3' and
reverse 5'-GCTCTCGATCAGCTTCACCAT-3' (reverse);
glutathione peroxidase-1 (GPX1),
forward, 5'-TCCACCGTGTATGCCTTCTCC-3' and
reverse, 5'-CCTGGTGTATCGCGCACTGA-3'; and
glyceraldehyde-3-phosphate dehydrogenase (GAPDH),
forward, 5'-TATGATGACATCAAGAAGGTGG-3' and
reverse, 5'-CACCACCTGTGCTGTA-3'.

PCR was performed for TRX1 by 5 min at 94 °C, followed by 41 cycles for 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, and 10 min at 72 °C. For GPX1, 31 cycles for 45 s at 94 °C, 1 min at 60 °C, 1.5 min at 72 °C, and 10 min at 72 °C. For GAPDH, 39 cycles for 45 s at 94 °C, 1 min at 60 °C, 1.5 min at 72 °C, and 10 min at 72 °C.

Five microliters of the PCR products were electrophoresed on 3% agarose gels. The gels stained with ethidium bromide were photographed with a UV transilluminator/digital imaging system (IS4000R; Eastman Kodak Company., New Haven, CT).

2.8. Cell cultures

The 661 W was provided by Dr. Muayyad R. Al-Ubaidi (University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA). The

661 W cells have been used to investigate photoreceptor cell death *in vitro*,¹¹ and they are sensitive to photo-oxidative stress similar to photoreceptor cells *in vivo*.¹² These cells were maintained in Dulbecco's modified Eagle medium (DMEM; Nacalai Tesque Inc, Kyoto, Japan) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Rockford, IL, USA), 100 U/mL of penicillin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and 100 µg/mL of streptomycin (Meiji Seika Pharma) under a humidified atmosphere of 5% CO₂ at 37 °C. The cells were passaged after trypsinization every 2 days.

2.9. L-buthionine-(S,R)-sulfoximine (BSO)/Glutamate cell death assay

The 661 W cells were seeded in 96-well plates at a density of 3.0×10^3 cells/well and incubated for 24 h under a humidified atmosphere of 5% CO₂ at 37 °C. Then, the cells were washed twice with DMEM and then immersed in DMEM supplemented with 1% FBS. One hour after pretreatment with 10, 50, or 100 µM of carteolol, 10, 50, or 100 µM of timolol or 1 mM of N-acetyl cysteine (NAC), 500 µM of L-buthionine-(S,R)-sulfoximine (BSO) and 10 mM of glutamate were added to the cell cultures for 24 h. NAC, an antioxidant, was used as a positive control. At the end of the culture period, 8.1 µM of Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) and 1.5 µM of propidium iodide (PI; Invitrogen) were added to the culture medium for 15 min. The rate of cell death was determined by double fluorescent staining with Hoechst 33342 and PI. The specimens were examined and photographed with a camera attached to an Olympus IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan).

2.10. Caspase-3/7 activation assay

Caspase-3/7 activity was measured at the end of the BSO/glutamate-induced cell death assay with the Caspase-Glo 3/7 Assay (Promega, Madison, WI) according to the manufacturer's instructions. After Caspase-Glo 3/7 assay reagent was added to each well in the 96-well plates, they were incubated at 37 °C for 1 h. The 96-well plates were loaded into a plate holder in a microplate reader (Varioskan Flash 2.4; Thermo Fisher Scientific, Waltham, MA, USA), and the luminescence was measured. The number of cells was determined by Hoechst 33342 staining, and the caspase-3/7 activity/cell was calculated.

2.11. Cellular reactive oxygen species (ROS) measurements

Intracellular radical activation was measured at the end of the BSO/glutamate-induced cell death assay with 5- (and 6) -chloromethyl-20,70-dichlorodihydrofluorescein diacetate, acethyl ester (CM-H₂DCFDA; Thermo Scientific). CM-H₂DCFDA was added to the culture medium at a final concentration of 10 µM. The 96-well plates were loaded into a fluorescence spectrophotometer (Variscan; Thermo Fisher Scientific). The reaction was carried out at 37 °C, and degree of fluorescence was measured at 488 nm excitation and 525 nm emission immediately and 1 h after adding the BSO/glutamate. The number of cells was determined by Hoechst 33342 staining and used to calculate ROS production/cell.

2.12. DPPH radical scavenging assay

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was dissolved in ethanol, and its radical scavenging activity was determined at different sample concentrations in 96-well plates. Ten microliters of the sample dissolved in ethanol and 190 µL of DPPH radical solution (0.1 mM) were added to each well. Ten microliters of ethanol and 190 µL of DPPH were used as the control group. After 30 min in

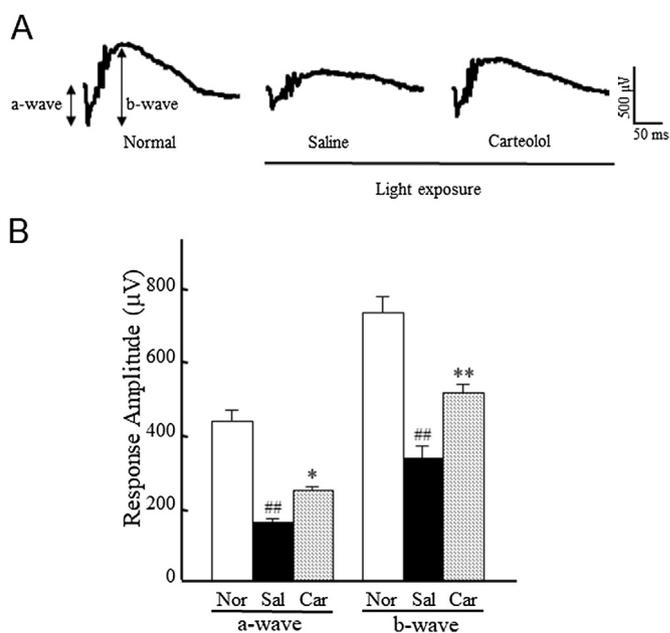


Fig. 1. Protective effects of carteolol against visible light-induced damage (8000 lux, 1 h) to the ERGs in pigmented rats. Carteolol or saline were applied topically to the eyes daily for 2 weeks before the light exposure. At 4 days after the light irradiation, the ERG recordings were conducted. (A) Typical traces of dark-adapted ERG responses. (B) Response amplitudes of a- and b-waves. The means of the a- and b-wave amplitudes obtained from both eyes were used for the statistical analyses. Normal represents non-irradiated rats without any treatment. Values are the means \pm standard error of the means (SEMs), $n = 4$. ##, $P < 0.01$ vs. Normal. *, $P < 0.05$ vs. Saline. **, $P < 0.01$ vs. Saline (Tukey–Kramer test, 2-sided). Nor, Normal; Sal, Saline; Car, carteolol.

a darkened room at room temperature, the absorbance was measured at 520 nm. Ethanol (50%) was used as the blank solution, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) diluted to 10, 50, 100, and 500 µM, was used as the positive control. The measurements were made in the same way for 0.1, 1, 10, 100, 1000 and 10,000 µM Carteolol-treated groups.

2.13. Lipid peroxidation in porcine retinal homogenates

Porcine retinal homogenates were prepared as described in detail.^{13,14} The retinal tissues were homogenized in a glass-Teflon homogenizer with 30% w/v of 1.15% ice-cold potassium chloride. The retinal homogenate was centrifuged at 3000 rpm for 10 min, and then 85 µL of the diluted homogenate was added to 10 µL of the sample (vehicle; 10, 100, 1000 or 10,000 µM carteolol; 1, 10 or 100 µM trolox) and 5 µL of 0.3 mM FeSO₄, and incubated for 1 h at 37 °C. The reaction was stopped by adding 20 µL of 35% HClO₄, and then centrifuged at 3000 rpm for 10 min at 10 °C. A 100 µL aliquot of the supernatant was heated with 20 µL of 8.1% sodium dodecyl sulfate (SDS) solution and 100 µL of 20% acetic acid buffer (pH 3.6) containing 0.8% thiobarbituric acid (TBA) solution at 100 °C for 1 h. After cooling, 200 µL of butyl alcohol and pyridine (15:1) was added, and the sample was gently shaken for 5 min. After centrifugation at 4000 rpm for 10 min, the butyl alcohol-pyridine phase containing the thiobarbituric acid reactive substance (TBARS) was separated, and its absorbance was measured at 532 nm.

2.14. Statistical analyses

The data are presented as the means \pm standard error of the means (SEMs). The statistical comparisons were determined by two-sided Student's *t* tests or one-way ANOVA followed by two-sided Tukey–Kramer tests or Dunnett's tests with the SPSS

Statistics (IBM, Armonk, NY, USA) software. A $P < 0.05$ was taken to be statistically significant.

3. Results

3.1. Retinal dysfunction and histological damages after light irradiation

To assess the retinal function, we conducted ERGs. In the saline-treated rats, there was a significant decrease in the a-wave ($161 \pm 12 \mu\text{V}$) and b-wave ($337 \pm 33 \mu\text{V}$) amplitudes after the light irradiation compared to that of the non-irradiated rats (a-wave, $438 \pm 34 \mu\text{V}$, $P < 0.001$; b-wave, $741 \pm 42 \mu\text{V}$, $P < 0.001$). In the carteolol-treated rats, the a- and b-wave amplitudes were significantly larger than those in the saline-treated rats (a-wave, $249 \pm 7 \mu\text{V}$, $P = 0.039$; b-wave, $518 \pm 21 \mu\text{V}$, $P = 0.010$) (Fig. 1).

Following the ERG recordings, the eyes were processed for conventional histological examinations to measure the ONL thickness. The ONL thickness in the saline-treated rats was markedly reduced compared to that in non-irradiated rats ($P < 0.001$). In contrast, the carteolol-treated rats significantly alleviated the thinning of the ONL compared to saline-treated rats ($P < 0.001$) (Fig. 2).

3.2. Oxidative stress induced by visible light

To investigate the cytoprotective mechanism of carteolol, the cells under photo-oxidative stress in the ONL of the rats pretreated with saline or carteolol were counted by immunostaining for

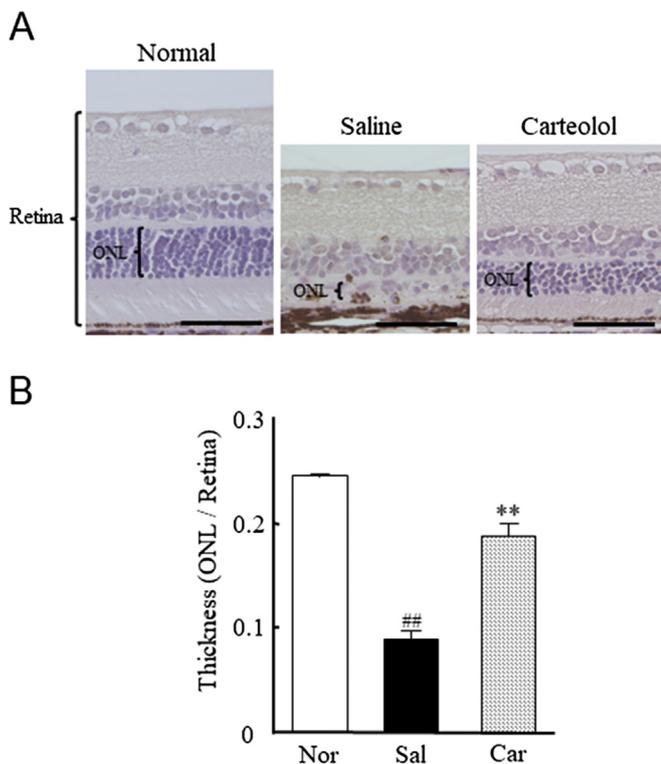


Fig. 2. Protective effects of carteolol against thinning of ONL by visible light irradiation (8000 lux, 1 h) in pigmented rats. (A) Representative photographs of hematoxylin and eosin staining of retinal sections after the ERG recordings. Scale bar: 50 μm . (B) Measurements of thickness of the ONL and the total retina. The thicknesses of the left and right eyes were averaged and used for the statistical analyses. The thicknesses of the ONL are shown as the ratio to the total retinal thickness. Normal represents non-irradiated rats without any treatment. Values are the means \pm SEMs, $n = 4$. ##, $P < 0.01$ vs. Normal. **, $P < 0.01$ vs. Saline (Tukey–Kramer test, 2-sided). Nor, Normal; Sal, Saline; Car, carteolol.

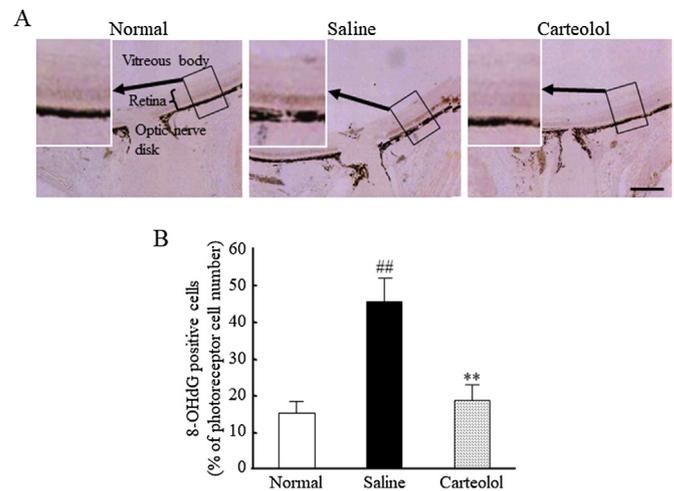


Fig. 3. Protective effects of carteolol on expression of 8-OHdG positive cells induced by exposure to visible light (8000 lux, 3 h) in pigmented rats. The test samples were topically applied to the eyes of rats for 2 weeks before the light exposure. Representative images for 8-OHdG immunostaining of posterior eyes (A) and the number of 8-OHdG positive cells (% of photoreceptor cell number) in the ONL (B) are shown. Dark brown-stained 8-OHdG positive cells can be seen in the ONLs of the saline-treated eyes. The mean value of both eyes was used for the statistical analyses. Normal represents non-irradiated rats without any treatment. Scale bar = 500 μm . Values are the means \pm SEMs, $n = 4$. ##, $P < 0.01$ vs. Normal. **, $P < 0.01$ vs. Saline (Tukey–Kramer test, 2-sided).

8-OHdG, a marker of oxidative-stress-induced DNA damage. The percentage of 8-OHdG-positive cells in the ONL of saline-treated rats was significantly increased immediately after light exposure as compared to the non-irradiated rats ($P < 0.001$). On the other hand, the percentage of 8-OHdG-positive cells in the carteolol-treated rats was significantly lower than that in the saline-treated rats ($P = 0.002$), and not significantly different from that in the non-irradiated rats ($P = 0.849$) (Fig. 3).

3.3. Expression of antioxidant enzymes in retinal tissue

To determine whether antioxidant enzymes were involved in the effects of carteolol in reducing the effects of oxidative stress, we analyzed the expression of the mRNAs of antioxidant enzymes including TRX1 and GPX1 by RT-PCR. The band intensity of TRX1 (2.0-fold and $P = 0.011$) and GPX1 (1.6-fold and $P = 0.012$) in the retinas from the carteolol-treated rats were significantly higher than that in the retinas from the saline-treated rats (Sup. Fig. 1 and Fig. 4).

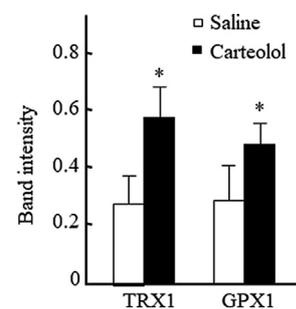


Fig. 4. RT-PCR analysis of the expression of the mRNAs of antioxidant enzymes in retinas from the mRNA expression of TRX1 and GPX1, antioxidant enzymes, in the retinal tissue of carteolol- or saline-treated dark-adapted rats. Band intensities were standardized with the band intensity of GAPDH. The full-length bands are presented in Supplemental Fig. 1. The means \pm SDs of the densities are shown in the bar graphs [$n = 5$ (rats) in each group]. *, $P < 0.05$ vs. Saline (Student's *t*-test, 2-sided). TRX1, thioredoxin-1; GPX1, glutathione peroxidase-1.

3.4. 661 W cell death induced by BSO/glutamate

It is known that BSO/glutamate induces oxidative stress which causes retinal cell death by the necrotic and/or apoptotic signaling pathways. Thus, we evaluated the effects of carteolol and timolol,

which is another β -adrenoceptor antagonist ophthalmic solutions and has neuroprotective effects previously reported,¹⁵ on the death of 661 W cells induced by BSO/glutamate. Exposure to 50 μ M ($P < 0.05$), 100 μ M ($P < 0.01$) carteolol and 100 μ M timolol ($P < 0.05$) significantly protected the 661 W cells against the BSO/

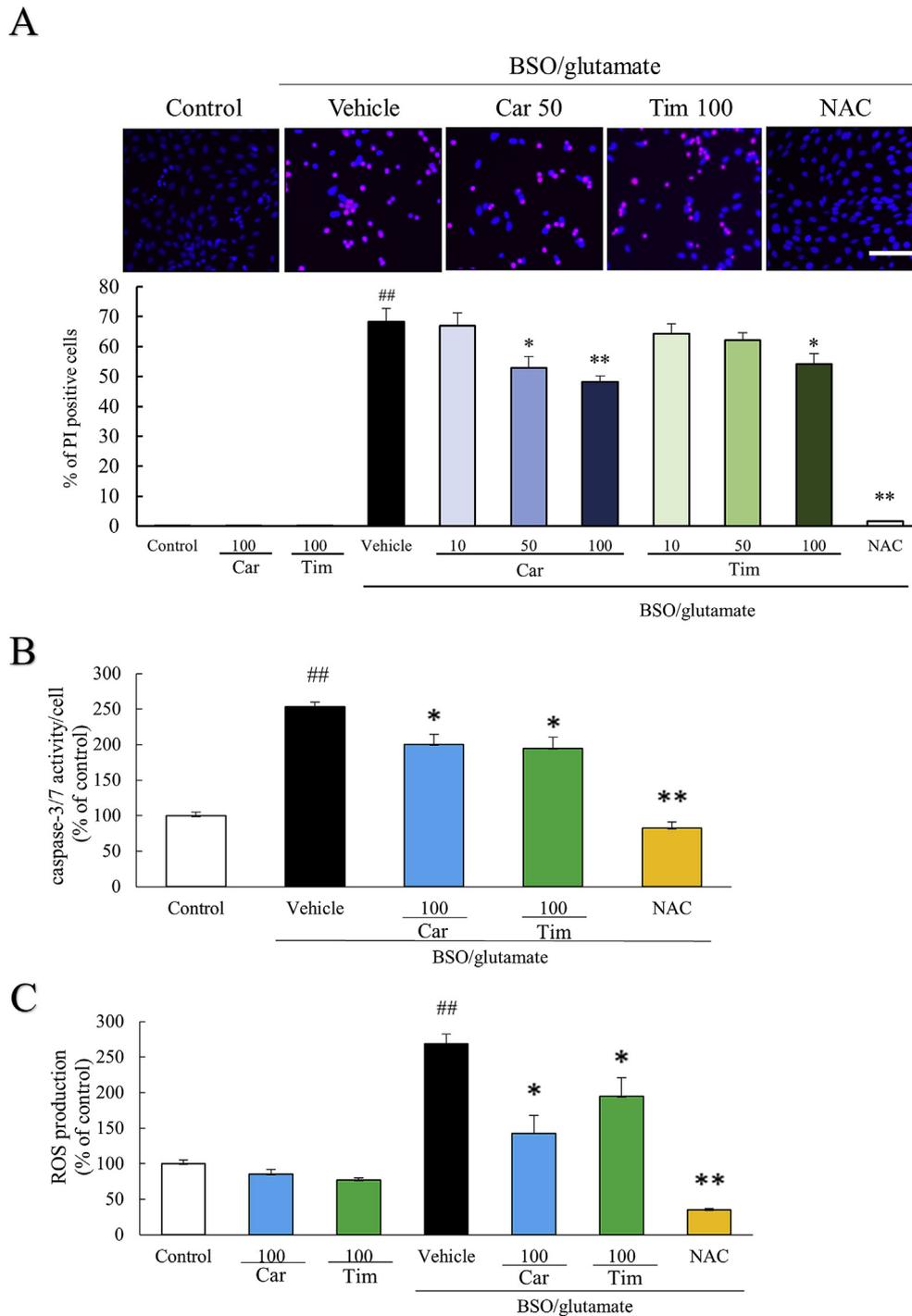


Fig. 5. Effects of carteolol and timolol against BSO/glutamate-induced oxidative stress on 661 W cells. **A:** Representative fluorescent photomicrographs of retinal sections stained by double fluorescent staining with Hoechst 33342 (Blue: dead and live cells) and propidium iodide (PI) (Red: dead cells). The total number of cells was counted in a masked manner, and the percentage of PI-positive cells was calculated. The evaluations of the treatment with carteolol and timolol against 661 W cell death induced by BSO/glutamate are shown. Treatment with carteolol at 50 μ M and at 100 μ M, and timolol at 100 μ M significantly protected the 661 W cells against BSO/glutamate-induced cell death. NAC was used for positive control. Scale bar = 100 μ m. Values are the means \pm SEMs. ($n = 6$). **B:** Evaluations of the effects of carteolol and timolol on caspase-3/7 activation in 661 W cells induced by BSO/glutamate. The caspase-3/7 activity was increased by BSO/Glutamate, but prior treatment of carteolol and timolol reduced the caspase-3/7 activation significantly. NAC was used for positive control. Values are the means \pm SEMs. ($n = 3$). **C:** Effects of carteolol and timolol on the BSO/glutamate induced production of reactive oxygen species (ROS) in 661 W cells. Carteolol at 100 μ M and timolol at 100 μ M significantly reduced the BSO/glutamate-induced ROS production in 661 W cells. NAC was used for positive control. Values are the means \pm SEMs. ($n = 6$) **##:** $P < 0.01$ vs. Control. *****; $P < 0.05$ vs. Vehicle. ******; $P < 0.01$ vs. Vehicle (Tukey–Kramer test, 2-sided). Car, Carteolol; Tim, Timolol; NAC, N-acetyl cysteine.

Glutamate-induced cell death (Fig. 5A). NAC (1 mM) also inhibited the cell death significantly ($P < 0.001$; Fig. 5A).

3.5. Caspase-3/7 activation and ROS production induced by BSO/glutamate

The caspase-3/7 activity was increased by BSO/glutamate but prior treatment with 100 μ M of carteolol and timolol reduced the degree of caspase-3/7 activation significantly ($P < 0.05$; Fig. 5B). The level of ROS was increased in 661 W cell cultures by BSO/glutamate, and 100 μ M of carteolol and timolol significantly reduced the ROS production ($P < 0.05$; Fig. 5C). NAC (1 mM) also reduced the BSO/glutamate toxicity (both, $P < 0.001$; Fig. 5B,C).

3.6. DPPH radical scavenging activity

To determine whether carteolol suppressed the production of ROS by radical scavenging directly, we examined the DPPH radical scavenging activity test. The results showed that carteolol didn't have direct DPPH radical scavenging ability. The antioxidant, Trolox was used for positive control (Sup. Fig. 2).

3.7. Production of TBARS

Lipid peroxidation has been reported to play an important role in degenerative ocular diseases.¹⁶ Therefore, we investigated the effects of carteolol against lipid peroxidation in porcine retinal homogenates. Carteolol didn't reduce the production of TBARS significantly. Trolox inhibited the production of TBARS in a concentration-dependent manner (Sup. Fig. 3).

4. Discussion

We found that the light-induced retinal damage was reduced significantly by the repeated instillation of carteolol before the irradiation (Figs. 1 and 2). Moreover, carteolol markedly inhibited the formation of 8-OHdG, a marker of oxidative stress-induced DNA damage, in the ONL (Fig. 3). These findings indicated that carteolol has physiologically and morphologically protective effects against photo-oxidative retinal damage. Furthermore, because carteolol didn't absorb visible light at wavelength ≥ 400 nm,⁷ a sunscreen-like action was unlikely to be responsible for the effects.

It was shown earlier that the \bullet OH scavenging ability of carteolol played an important role in protecting the cornea from UV irradiation.⁶ Additionally, carteolol had the ability (ED₅₀ value: 48 mM) to scavenge O₂⁻, a form of ROS, and prevented corneal epithelial cells from being damaged by hydrogen peroxide (H₂O₂).⁷ Moreover, the concentration plateau of topical 2% ¹⁴C-carteolol solution application twice/day in the rabbits' retina and choroid tissues was attained with approximately 30 μ M after more than 20 instillations.⁸ Based on it, the carteolol concentration in the retinal and choroidal tissues should have reached a plateau after 2 weeks of twice/day instillation of 2% carteolol solution. Thus, the carteolol concentration which possessed the scavenging ability (ED₅₀ value: 48 mM)⁷ was much higher than the concentration attained with repeated instillations of 2% carteolol (30 μ M).⁸ Furthermore, carteolol up to 10 mM didn't have the ability to scavenge the DPPH radicals (Sup. Fig. 2) and didn't reduce the production of TBARS significantly (Sup. Fig. 3). Therefore, we suggest that the neuroprotective function of carteolol against photo-oxidative stress would have resulted from multiple indirect effects but not from direct radical scavenging ability.

The expressions of the mRNA of TRX1 and GPX1 were significantly increased in the retinal tissues after 2 weeks of twice/day instillation of carteolol (Fig. 4). Both proteins are located in the

retinal photoreceptor layers and the retinal pigment epithelium, and they act as protective agents against photo-oxidative stress.^{17,18} The results suggested that the TRX intensification might be a useful therapeutic strategy to prevent light-induced retinal damage.¹⁷ Thus, the neuroprotective effect of carteolol might come from enhanced antioxidative potential by inducing these endogenous antioxidative proteins.

Both carteolol and timolol inhibited the BSO/glutamate-induced photoreceptor cell death, suppressed the caspase-3/7 activity/cell, and reduced the production of ROS (Fig. 5). The level of caspase-3/7 was elevated in the 661 W cells damaged by oxidative stress,¹⁹ which played an important role in the apoptosis of the cells.²⁰ In addition, the oxidative stress induced by excessive ROS may be involved in the photoreceptor cell death.^{21,22} These results indicated that carteolol and timolol could have possessed the neuroprotective effect with reduction of intracellular ROS production through beta-adrenoceptor. Taking into consideration the neuroprotective effects of carteolol and timolol were observed only at a very high dose *in vitro*, the effects should have resulted from not only the action through β -adrenoceptors but also the multiple effects.

Carteolol has intrinsic sympathetic activity, however timolol doesn't have. Therefore, timolol would have shown the protective effect by the β -adrenoceptor blockade, while carteolol might have shown by the partial stimulation of the β -adrenoceptors because endogenous catecholamines would not exist or exist in very low concentrations *in vitro* situation. While there are only a few reports investigating intrinsic sympathetic activity of carteolol eye drops,²³ we also believe that its actual clinical impact is very small.

It is well known that photo-oxidative stress is involved in the pathogenesis and exacerbation of AMD.¹ Additionally, there are other common ocular diseases (dry eye syndrome; corneal and conjunctive diseases; cataract; glaucoma; retinitis pigmentosa; diabetic retinopathy, autoimmune and inflammatory uveitis) associated with oxidative stress.²⁴ Our findings suggest that carteolol might suppress the development and aggravation of these diseases.

In conclusion, carteolol application alleviated the retinal damage caused by light-exposure *in vivo*, and photoreceptor cell death and apoptosis stimulated by BSO/glutamate-induced oxidative stress *in vitro*. The neuroprotective effects could have resulted from the multiple effects such as enhancing the antioxidative potential by inducing TRX1 and GPX1 and decreasing the intracellular ROS production through β -adrenoceptors.

Conflict of interest

The authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jphs.2018.11.010>.

References

- Beatty S, Koh H, Phil M, Henson D, Boulton M. The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol.* 2000;45(2): 115–134.
- Sacca SC, Roszkowska AM, Izzotti A. Environmental light and endogenous antioxidants as the main determinants of non-cancer ocular diseases. *Mutat Res.* 2013;752(2):153–171.
- Otsuka T, Shimazawa M, Nakanishi T, et al. Protective effects of a dietary carotenoid, astaxanthin, against light-induced retinal damage. *J Pharmacol Sci.* 2013;123(3):209–218.
- Wong P, Markey M, Rapp CM, Darrow RM, Ziesel A, Organisciak DT. Enhancing the efficacy of AREDS antioxidants in light-induced retinal degeneration. *Mol Vis.* 2017;23:718–739.

5. Frishman WH, Fuksbrumer MS, Tannenbaum M. Topical ophthalmic beta-adrenergic blockade for the treatment of glaucoma and ocular hypertension. *J Clin pharmacol*. 1994;34(8):795–803.
6. Tanito M, Takanashi T, Kaidzu S, Yoshida Y, Ohira A. Cytoprotective effects of rebamipide and carteolol hydrochloride against ultraviolet B-induced corneal damage in mice. *Invest Ophthalmol Vis Sci*. 2003;44(7):2980–2985.
7. Kuwahara K, Oizumi N, Fujisawa S, Tanito M, Ohira A. Carteolol hydrochloride protects human corneal epithelial cells from UVB-induced damage in vitro. *Cornea*. 2005;24(2):213–220.
8. Fujio N, Kusumoto N, Odomi M. Ocular distribution of carteolol after single and repeated ocular instillation in pigmented rabbits. *Acta Ophthalmol*. 1994;72(6):688–693.
9. Hollo G, Whitson JT, Faulkner R, et al. Concentrations of betaxolol in ocular tissues of patients with glaucoma and normal monkeys after 1 month of topical ocular administration. *Invest Ophthalmol Vis Sci*. 2006;47(1):235–240.
10. Tanito M, Kaidzu S, Ohira A, Anderson RE. Topography of retinal damage in light-exposed albino rats. *Exp Eye Res*. 2008;87(3):292–295.
11. Tanito M, Kwon YW, Kondo N, et al. Cytoprotective effects of geranylgeranylacetone against retinal photooxidative damage. *J Neurosci*. 2005;25(9):2396–2404.
12. Krishnamoorthy RR, Crawford MJ, Chaturvedi MM, et al. Photo-oxidative stress down-modulates the activity of nuclear factor-kappaB via involvement of caspase-1, leading to apoptosis of photoreceptor cells. *J Biol Chem*. 1999;274(6):3734–3743.
13. Inokuchi Y, Shimazawa M, Nakajima Y, Suemori S, Mishima S, Hara H. Brazilian green propolis protects against retinal damage in vitro and in vivo. *ECAM*. 2006;3(1):71–77.
14. Izawa H, Shimazawa M, Inoue Y, et al. Protective effects of NSP-116, a novel imidazolyl aniline derivative, against light-induced retinal damage in vitro and in vivo. *Free Radic Biol Med*. 2016;96:304–312.
15. Goto W, Ota T, Morikawa N, et al. Protective effects of timolol against the neuronal damage induced by glutamate and ischemia in the rat retina. *Brain Res*. 2002;958(1):10–19.
16. Njie-Mbye YF, Kulkarni-Chitnis M, Opere CA, Barrett A, Ohia SE. Lipid peroxidation: pathophysiological and pharmacological implications in the eye. *Front Physiol*. 2013;4:366.
17. Tanito M, Masutani H, Nakamura H, Ohira A, Yodoi J. Cytoprotective effect of thioredoxin against retinal photic injury in mice. *Invest Ophthalmol Vis Sci*. 2002;43(4):1162–1167.
18. Ohira A, Tanito M, Kaidzu S, Kondo T. Glutathione peroxidase induced in rat retinas to counteract photic injury. *Invest Ophthalmol Vis Sci*. 2003;44(3):1230–1236.
19. Sanvicens N, Gomez-Vicente V, Masip I, Messeguer A, Cotter TG. Oxidative stress-induced apoptosis in retinal photoreceptor cells is mediated by calpains and caspases and blocked by the oxygen radical scavenger CR-6. *J Biol Chem*. 2004;279(38):39268–39278.
20. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. *CSH Perspect Biol*. 2015;7(4):a026716.
21. Yang JH, Basinger SF, Gross RL, Wu SM. Blue light-induced generation of reactive oxygen species in photoreceptor ellipsoids requires mitochondrial electron transport. *Invest Ophthalmol Vis Sci*. 2003;44(3):1312–1319.
22. Roehlecke C, Schumann U, Ader M, et al. Stress reaction in outer segments of photoreceptors after blue light irradiation. *PLoS One*. 2013;8(9):e71570.
23. Brooks AM, Gillies WE. Ocular beta-blockers in glaucoma management. Clinical pharmacological aspects. *Drugs Aging*. 1992;2(3):208–221.
24. Kruk J, Kubasik-Kladna K, Aboul-Enein HY. The role oxidative stress in the pathogenesis of eye diseases: current status and a dual role of physical activity. *Mini Rev Med Chem*. 2015;16(3):241–257.