Glaucoma is a progressive neurodegenerative disease affecting over 60 million people worldwide and predicted to affect more than 80 million people by 2020. Glaucoma is also the second most common cause of irreversible blindness. An increase in intraocular pressure (IOP) contributes to the pathogenesis of glaucoma. Treatment of glaucoma is focused on decreasing IOP, because IOP is one of the most important risk factors for disease progression. Glaucoma drugs that reduce IOP include beta blockers, carbonic anhydrase inhibitors, prostaglandin analogs, sympathomimetics, and miotics. Retinal ganglion cells are highly sensitive to increased IOP, and retinal ganglion cell damage is caused by several types of cell stress. For example, retinal ganglion cell death in glaucoma is not only related to oxidative stress, but also to endoplasmic reticulum (ER) stress and ischemic stress. Moreover, a previous report suggested that this retinal ganglion cell death is mediated by phosphatidyl inositol 3-kinase (PI3K)/Akt pathway, and the inhibition of PI3K/Akt pathway promoted retinal ganglion cell survival in rat optic nerve injury model. Many patients with glaucoma have normal IOP. Therefore, it is important to not only decrease IOP, but also to protect retinal cells during glaucoma therapy.

Prostaglandin analogs, such as latanoprost, are the major therapy that reduces IOP by stimulating drainage of the aqueous humor. Latanoprost is a prostaglandin F2α analog and is effective at reducing IOP when used alone. Additionally, latanoprost protects the primary retinal ganglion cells, independent of the IOP reduction, by stimulating glutathione and reducing reactive oxygen species.

Beta blockers, such as timolol, are widely used in the treatment of glaucoma. Monotherapy with timolol decreases aqueous humor production to reduce IOP, and protects retinal ganglion cells in a rat glaucoma model. Latanoprost and timolol in a fixed combination greatly reduced IOP in patients with primary open angle glaucoma in a double masked, randomized clinical trial. The latanoprost/timolol combination provides a greater ocular hypotensive effect than monotherapy with latanoprost or timolol. Although the greater ocular hypotensive effect of combination is well-understood, it is not known if the combination has a greater neuroprotective effect than either drug alone.

In the present study, we have investigated the neuroprotective effects of a latanoprost/timolol combination on retinal damage and the mechanism of the protective effect of this combination of drugs in vitro.

MATERIALS AND METHODS

Materials Latanoprost was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.) and dissolved in dimethyl sulfoxide. Timolol was obtained from LKT Laboratories (St. Paul, MN, U.S.A.). Hydrogen peroxide, tunicamycin and buthionine sulfoximine (BSO) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glutamate was obtained from Nacalai Tesque (Kyoto, Japan). The PI3 kinase inhibitor 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, LY294002, was obtained from Cell Signaling Technology (Danvers, MA, U.S.A.).

Neuroretinal Cells Neuroretinal cells (RGC-5) were a kind gift from Dr. Neeraj Agarwal (University of North Texas Health Science Center, Fort Worth, TX, U.S.A.). Cultures of RGC-5 were maintained in 1 mg/mL glucose Dulbecco’s modified Eagle’s medium (DMEM; Nacalai) containing 10% fetal bovine serum (FBS, Lot number; 31K8461), 100 U/mL...
penicillin (Meiji Seika, Tokyo, Japan), and 100 µg/mL streptomycin (Meiji Seika) and were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. In all experiments, we seed RGC-5 at a density of 1.0×10³ cells per well with DMEM containing 10% FBS in 96 well plates, and then incubated for 24h.

**Oxidative Stress-Induced Cell Death** The medium was changed to DMEM containing 1% FBS and the cells were treated with single drugs (timolol or latanoprost; 1, 10, 100, 1000 nM) or a latanoprost/timolol combination (1 nM latano-
prost/1, 10, 100, 1000 nM timolol; 1 nM timolol/1, 10, 100, 1000 nM latanoprost; 1000 nM latanoprost/100 nM timolol) for 1 h. Trolox, which is a water-soluble analog of vitamin E, was used as a positive control. Hydrogen peroxide was added at a final concentration of 0.3 mM to each well for 27 h after which the cells were analyzed by a nuclear staining assay.

Serum Deprivation-Induced Cell Death The cells were washed three times and the medium was changed to serum-free DMEM. The cells were then treated with single drugs (timolol or latanoprost; 1, 10, 100, 1000 nM) or a latanoprost/timolol combination (1 nM latanoprost/1, 10, 100, 1000 nM timolol; 1 nM timolol/1, 10, 100, 1000 nM latanoprost). Trolox was used as a positive control. Nuclear staining assay was carried out after 48 h.

Endoplasmic Reticulum Stress-Induced Cell Death The medium was changed to DMEM containing 1% FBS and the cells were treated with a single drug (timolol or latanoprost; 0.1, 1, 10, 100 nM) or a latanoprost/timolol combination (0.1 nM latanoprost/0.1, 1, 10, 100 nM timolol; 0.1 nM timolol/0.1, 1, 10, 100 nM latanoprost) for 1 h. Then, tunicamycin (a final concentration of 2 µg/mL) was added to each well, the cells incubated for 27 h, and the cells were analyzed by nuclear staining.

Effect of an Akt Inhibitor on Serum Deprivation-Induced Cell Damage The cells were then washed three times and the medium was changed to serum-free DMEM with or without 0.3 µM LY294002. One hour after adding LY294002, the cells were treated with a single drug (1 nM timolol and 1000 nM latanoprost) or the 1000 nM latanoprost/1 nM timolol combination for 27 h. The cells were then analyzed for nuclear staining.

Effect of an Akt Inhibitor on BSO Plus Glutamate-Induced Cell Damage The medium was changed to DMEM containing 1% FBS and the cells were treated with a latanoprost/timolol combination (1 nM latanoprost/1 nM timolol) for 1 h. Then, BSO (0.05 mM)/glutamate (1 mM) was added to each well for 24 h and the cells in 96-well plates were analyzed for nuclear staining.

Nuclear Staining Assay Cell death was observed by double staining with 2 fluorescent dyes, Hoechst 33342 (Invitrogen, Carlsbad, CA, U.S.A.) and propidium iodide (PI) (Invitrogen). At the end of the experiment, Hoechst 33342 and PI were added to the culture medium for 15 min at final concentrations of 8.1 and 1.5 µM, respectively. Images were collected using an Olympus IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan). The total number of cells was counted in a blind manner (by S.F.) and the percentage of

**Fig. 2. Latanoprost/Timolol in Combination Have a Protective Effect on ER Stress**

(A) Monotherapy with latanoprost at concentrations above 1.0 nM reduced the number of dead cells. (B) Monotherapy with timolol at concentrations above 1.0 nM also reduced cell death. (C) Latanoprost (0.1 nM) and timolol (0.1, 1, 10, 100, and 1000 nM) in combination reduced the number of dead cells and latanoprost (0.1 nM) and timolol (10 and 100 nM) in combination showed a stronger protective effect on ER stress than monotherapy with 0.1 nM latanoprost alone. (D) Timolol (1 nM) and latanoprost (1, 10, 100, and 1000 nM) in combination also reduced cell death and timolol (1.0 nM)/latanoprost (10 and 100 nM) showed a stronger protective effect on ER stress than monotherapy with timolol. The data are expressed as the mean±S.E.M. (n=6 to 12). ****p<0.01 vs. Control alone; *p<0.05, **p<0.01 vs. Vehicle; +p<0.05, ++p<0.01 vs. exposed to tunicamycin and treated by 0.1 nM latanoprost or timolol.
Pl-positive cells was calculated.

**Statistical Analysis** Data are presented as the mean±standard error of the mean (S.E.M.). Statistical comparisons were made using Student’s *t*-test or ANOVA with Dunnett’s test using STATVIEW version 5.0 (SAS Institute, Cary, NC, U.S.A.). *p*<0.05 was considered to indicate statistical significance.

**RESULTS**

**Protective Effects of Latanoprost/Timolol Combination on Cell Death Induced by Oxidative Stress** A previous study has suggested that oxidative stress is strongly associated with glaucomatous retinal cell death.5) We investigated the cytoprotective effects of latanoprost, timolol, and latanoprost/timolol in combination on oxidative stress-induced cell damage induced by hydrogen peroxide. Oxidative stress increased PI-positive cells, an effect that was reduced by timolol and latanoprost and reduced PI-positive cells (Fig. 1A). Trolox, used as a positive control, significantly reduced cell death (Figs. 1B, C). Treatment with latanoprost at concentrations from 10–1000 nM reduced hydrogen peroxide-induced cell death in a concentration-dependent manner (Figs. 1B, C). Trolox, used as a positive control, significantly reduced cell death (Figs. 1B, C). However, monotherapy with latanoprost at 1 nM did not show a neuroprotective effect (Figs. 1B, C), and toxicity was observed after monotherapy with latanoprost or timolol at concentrations above 1000 nM (data not shown).

Latanoprost at 1 nM in combination with timolol at 10, 100, or 1000 nM provided a greater protective effect than latanoprost alone (Fig. 1D). Similarly, timolol at 1 nM along with latanoprost at 10, 100, or 1000 nM reduced cell death more than timolol alone (Fig. 1E). Furthermore, although monotherapy with latanoprost or timolol at 1 nM did not show a protective effect, in combination there was a remarkable reduction in the number of dead cells (Figs. 1D, E).

**Protective Effects of Timolol/Latanoprost in Combination on Cell Death Induced by Endoplasmic Reticulum Stress** Previous reports have shown that ER stress-induced retinal ganglion cell death is via the phosphorylated eukaryotic initiation factor 2 (C/EBP)-homologous protein (PERK-p-eIF2alpha-CHOP) pathway in a chronic glaucoma model.14) We investigated whether latanoprost or timolol has a protective effect on ER stress. Tunicamycin induces ER stress by blocking the biosynthesis of N-linked oligosaccharides and increases cell death.15) Monotherapy with latanoprost or timolol at 1, 10, and 100 nM, decreased cell death induced by ER stress in a concentration-dependent manner (Figs. 2A, B). Although monotherapy with latanoprost or timolol at 0.1 nM did not show a protective effect, latanoprost or timolol at 1 nM reduced cell death (Figs. 2C, D). Latanoprost at 0.1 nM along with timolol at 0.1, 1, 10,
or 100 nM decreased the cell death. Latanoprost at 0.1 nM with timolol at 10 or 100 nM had a greater protective effect than monotherapy with latanoprost at 0.1 nM (Fig. 2C). Timolol at 0.1 nM combined with latanoprost at 1, 10, 100, or 1000 nM decreased cell death, and all of the combinations had a stronger protective effect than monotherapy with timolol at 0.1 nM.

**Protective Effects of Timolol/Latanoprost Combination on Cell Death Induced by Serum Deprivation**

Several reports have shown that ischemic stress causes significant retinal ganglion cell death in primary open angle glaucoma. 16,17) We have cultured cells in serum-free medium as a model of growth factor deprivation. 18) Serum deprivation significantly increased cell death. Monotherapy with latanoprost at 1, 10, 100, or 1000 nM had no protective effect (Fig. 3A), although timolol at 10, 100, and 1000 nM decreased cell death induced by serum deprivation (Fig. 3B). Timolol at 1 nM in combination with latanoprost at 1, 10, 100, or 1000 nM decreased cell death, and showed a greater protective effect than monotherapy with timolol at 1 nM.

**Inhibition of the Protective Effect of Timolol/Latanoprost in Combination by an Akt Inhibitor on Cell Death Induced by Serum Deprivation or BSO Plus Glutamate**

LY294002 was used to inhibit protein kinase B (Akt) inhibitor.19) As shown above, latanoprost at 1000 nM combined with timolol at 1 nM has a strong protective effect on cell death induced by serum deprivation. LY294002 added to this combination significantly increased cell death (Fig. 4A).

Latanoprost at 0.1 nM with timolol at 0.1 nM also reduced cell death produced by BSO plus glutamate. BSO plus glutamate induces oxidative stress; BSO depletes intracellular glutathione (GSH), and glutamate inhibits cystine uptake.20,21) LY294002 inhibited the protective effect of the latanoprost/timolol combination against BSO plus glutamate-induced cell death, as shown by an increase in the amount of cell death (Fig. 4B).

**DISCUSSION**

In the present study, latanoprost and timolol showed protective effects against several stressors, and the latanoprost/timolol combinations showed greater neuroprotective effects than latanoprost or timolol alone.

Oxidative stress is strongly related to glaucomatous cell death induced by ocular hypertension. 22–24) In the present study, we investigated the effect of latanoprost/timolol in combination on oxidative stress induced by hydrogen peroxide. Monotherapy with latanoprost or timolol and the latanoprost/timolol combination showed a protective effect on cell death. The latanoprost/timolol combination had a greater protective...
effect than monotherapy with latanoprost or timolol. Monotherapy protected against oxidative stress in a concentration-dependent manner, but high concentration of latanoprost and timolol showed neurotoxicity (data not shown). Moreover, latanoprost/timolol combined had a more potent protective effect than when given alone. Taken together, these findings indicate that latanoprost/timolol in combination could have a higher therapeutic value and fewer side effects than monotherapy with latanoprost or timolol.

ER stress is related to retinal ganglion cell damage and primary open angle glaucoma. In our previous reports, ER stress, which activates the BH3 interacting domain death agonist (Bid) and caspase-2, and initiates apoptotic cell death, occurs after elevation of IOP. Moreover, in the experimental glaucoma model, ER stress also induces retinal cell death thorough activation of N-methyl d-aspartate (NMDA) receptors. Tunicamycin, which blocks the formation of dolichol-mediated protein glycosylation, is used as an ER stress inducer. Monotherapy with latanoprost or timolol at concentrations from 1 to 1000 nM protected against cell death induced by tunicamycin. Latanoprost at 0.1 nM or timolol at 0.1 nM did not have a protective effect, whereas latanoprost at 0.1 nM/timolol at 0.1 nM in combination protected the cells. The latanoprost/timolol combination protected against ER stress to a greater extent than the monotherapy. These data also suggest that the therapeutic effect of the latanoprost/timolol combination shows a higher neuroprotective effect in comparison to monotherapy with latanoprost or timolol.

Serum deprivation, which induces apoptosis in retinal cells by causing an increase of reactive oxygen species through mitochondrial signaling pathways, correlates with retinal cell death in glaucoma. In the present study, we have investigated whether latanoprost/timolol in combination has a protective effect on cell death induced by serum deprivation (Fig. 3). Timolol (10, 100, and 1000 nM) significantly protected against the adverse effects of serum deprivation. In contrast, latanoprost had no protective effect on serum deprivation-induced cell damage. Beta blockers, such as timolol and metipranolol, suppress Ca\(^{2+}\) influx through voltage-sensitive channels. Consequently, ischemic stress was hypothesized to be reduced by beta blockers, thereby promoting cell survival. Serum deprivation has been used as a model of ischemic stress, therefore, it was thought that timolol would protect against serum deprivation-induced stress through the reduction of Ca\(^{2+}\) influx. Timolol had a protective effect on serum deprivation-induced cell damage, but latanoprost did not. In a previous study, we found that bimatoprost, which is also a prostaglandin analog, protected against serum deprivation-induced cell death. This difference of protective effects between latanoprost and bimatoprost might relate to receptor affinity or from activation of other protective pathways. Although monotherapy with latanoprost or timolol at 1 nM had no protective effect against serum deprivation, timolol at 1 nM and latanoprost (1, 10, 100, and 1000 nM) significantly protected against all damage. These findings indicate that latanoprost and timolol in combination have stronger therapeutic effects against neuroretinal cell (RGC-5) damage induced by serum deprivation.

Some previous reports have indicated that the PI3K/Akt pathway plays pivotal roles in cell viability after elevation of IOP, and retinal ganglion cell death in glaucoma is closely associated with the PI3K/Akt pathway. We used LY294002 to inhibit Akt and to investigate the mechanism of protective effect of latanoprost/timolol in combination. LY294002 specifically inhibits PI3K. The latanoprost/timolol combination protected against serum deprivation-induced cell damage. LY294002 added to cells that had been exposed to latanoprost at 1000 nM with timolol at 1 nM blocked the protective effect of the combination against serum deprivation. Furthermore, LY294002 inhibited the protective effect of the combination on BSO plus glutamate-induced cell death. Thus, the protective effects of latanoprost/timolol in combination might be associated with the PI3K/Akt pathway under conditions of ischemic stress and oxidative stress.

Oxidative stress, ER stress, and serum deprivation stress promote apoptosis via the reduction of the Akt/PI3K pathway. Taken together, the present findings suggest that activation of the PI3K/Akt pathway by the latanoprost/timolol combination contributes to its protective effect.

In conclusion, low-concentrated timolol and latanoprost combination which has no protective effects in monotherapy have protective effect against some stresses. Therefore, latanoprost and timolol in combination have more potent protective effects against RGC-5 damage induced by oxidative stress, serum deprivation stress, or ER stress than monotherapy with latanoprost or timolol. One of the mechanisms of this combination might be associated with PI3K/Akt pathway.

Conflict of Interest We received financial support from Pfizer (Tokyo, Japan), as collaborative research.

REFERENCES


10) Osborne NN, Ji D, Abdul Majid AS, Fawcett RJ, Sparatore A, Del Soldato P. ACS67, a hydrogen sulfide-releasing derivative of latano-


