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KR-31378, a Potassium-Channel Opener, Induces the Protection of Retinal Ganglion Cells in Rat Retinal Ischemic Models

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Abstract. KR-31378 is a newly developed K_{ATP}-channel opener. To investigate the ability of KR-31378 to protect retinal ganglion cells (RGC), experiments were conducted using two retinal ischemia models. Retinal ischemia was induced by transient high intraocular pressure (IOP) for acute ischemia and by three episcleral vein occlusion for chronic retinal ischemia. KR-31378 was injected intraperitoneally and administered orally in the acute and chronic ischemia models, respectively. Under the condition of chronic ischemia, RGC density in the KR-31378–treated group was statistically higher than that in the non-treated group, and IOP was reduced. In the acute retinal ischemia model, 90% of RGC were degenerated after one week in non-treated retina, but, RGC in KR-31378–treated retina were protected from ischemic damage in a dose-dependent manner and showed inhibited glial fibrillary acidic protein (GFAP) expression. Furthermore, the KR-31378 protective effect was inhibited by glibenclamide treatment in acute ischemia. These findings indicate that systemic KR-31378 treatment may protect against ischemic injury–induced ganglion cell loss in glaucoma.

Keywords: KR-31378, retinal ganglion cell, retinal ischemia, intraocular pressure, glaucoma

Introduction

Glaucoma is characterized by a slow progressive degeneration of retinal ganglion cells (RGCs) and their axons, which is usually associated with elevated intraocular pressure (IOP) (1 – 3). Common adult-onset glaucoma is primary open-angle glaucoma (POAG), which is probably caused by a reduction in outflow of aqueous humor through the trabecular outflow pathways. Normal tension glaucoma (NTG), a subset of POAG that presents statistically normal IOP, also shows glaucomatous optic neuropathy and relevant visual field defect. IOP still seems to play a role in NTG, since a substantial number of patients with NTG as well as other forms of POAG benefit from the lowering of IOP (3, 4).

In ischemic retinal damage, RGC degenerate and vision is lost due to excitotoxicity. In cells damaged by ischemic neuronal injury, many programmed cell death genes are expressed, including p53, caspase-3, COX-2 (cycloxygenase-2), and nNOS (neuronal nitric oxide synthesis) (5). In addition, glial cell activation is observed, for example, expression of glial fibrillary acidic protein (GFAP) and vimentin increased in glial cells (6, 7). In the retina, Müller gilal cells regulate ion exchange and glutamate uptake (8).

Ischemic retinal injury induces RGC death due to glutamate neurotoxicity. Glutamate uptake is mainly regulated by glutamate/aspartate transporter (GLAST), a glial-type glutamate transporter expressed in Müller cells (9). Harada et al. recently reported that GLAST knockout mice show NTG-like phenotypes, such as RGC loss and optic neuropathy, under the condition of normal IOP (3, 9). These results suggest that ischemia-induced glutamate neurotoxicity is involved in various types of glaucoma including NTG.

ATP-sensitive potassium channels (K_{ATP} channels) in the mitochondrial or plasma membrane of retinal cells may provide protection against retinal ischemia (10, 11). Yamauchi and colleagues suggested that opening of mitochondrial K_{ATP} channels can inhibit glutamate. Skatchkov and his colleagues observed K_{ATP} channels in Müller cells and suggested that these channels regulate

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retinal current and play a key role in retinal protection against ischemic conditions (11).

K_{ATP} channels play an important role in enhancing the resistance of the retina against ischemic insult (10 – 12) and are involved in an endogenous protective mechanism against ischemia–reperfusion injury in the heart and brain (13 – 15). This protective effect may function by the opening of K_{ATP} channels, which hyperpolarizes the plasma membrane and reduces the influx of calcium ions. K_{ATP}-channel openers, diazoxide and iptakalim showed neuroprotective effects in brain ischemia (16, 17).

In other studies, Maxi-K channels and K_{ATP} channels were observed in ciliary muscles and the trabecular meshwork in eyes (18 – 21), and it was postulated that opening of these potassium channels may increase uveal outflow.

KR-31378, (2*S*,3*S*,4*R*)-*N*''-cyano-*N*(6-amino-3,4-dihydro-3-hydroxy-2-methyl-2-dimethoxymethyl-2*H*-benzopyran-4-yl)-*N*'-benzylguanidine, is a newly developed benzopyran analog synthesized by the Korea Research Institute of Chemical Technology (Daejeon, Korea) (22). It was reported to be a potent K_{ATP}-channel opener and it exhibited an antioxidant effect and anti-apoptotic activities against ischemic brain injuries in rats in vitro (23 – 25).

In the present study, we investigated the effect of KR-31378 on reducing IOP and its protective effect on RGC against cell death, under conditions of chronic ocular hypertension. We also studied the protective effect of KR-31378 on RGC in acute ischemic injury to investigate retinal neuroprotective effects of KR-31378 independently from its IOP-lowering effect.

Materials and Methods

Chronic hypertensive glaucomatous model and drug administration

Six-week-old Sprague-Dawley rats (200 g, 50 animals; Orient Bio, Inc., Seongnam, Korea) were used (n = 10). All animal procedures conformed to the guidelines of Catholic University of Korea and *Principles of Laboratory Animal Care* (NIH publication No. 85-3, revised 1985). Rats were anesthetized by intra-peritoneal injection of a mixture of ketamine hydrochloride (20 mg/kg) and xylazine hydrochloride (2.5 mg/kg), both obtained from Yuhan Corp. (Seoul, Korea). The three episcleral (dorsal, ventral, and temporal) veins of the right eye were cauterized by hand-held cautery (Change-A-Tip™; Aaron, St. Petersburg, FL, USA), while the left eye did not receive surgical procedures and served as a control. IOP was measured 24 h following cautery and each morning thereafter with a Tono-Pen (Medtronic Solan, Jacksonville, FL, USA). The IOP measurements were made 5 times/day in one eye. And the IOP in each group expressed as mean IOP (mean ± S.E.M.). Animals in which the cauterized, hypertensive eye maintained a pressure of 28 – 31 mmHg for 7 days following surgery were selected as models of hypertensive glaucoma. Seventy percent of animals receiving vein occlusion surgery were satisfactory for models of hypertensive glaucoma. KR-31378 administration was initiated 7 days after surgery, which was considered treatment day zero.

KR-31378 dissolved in saline (0.9% sodium chloride in distilled water) was delivered twice per day (9 am and 9 pm). Drug administration in one of three doses (5, 10, or 30 mg/kg) was performed orally by tube. The animal weight was checked every day during the drug administration period. All experimental animals given KR-31378 were healthy, and the weights of the animals were not decreased during the experimental period.

Measurement of RGC density

Six weeks after initiating KR-31378 treatment, 4-[4-didecylaminostyryl]-*N*-methylpyridinium iodide (4Di-10ASP; Molecular Probe, Eugene, OR, USA) was injected into the left superior colliculus by using a stereotaxic instrument fitted with a micro-injector (Stoelting, Wood Dale, IL, USA). Five days after injection of 4Di-10ASP, the rats were sacrificed and their eyes were enucleated. The retinas were separated from the choroids and sclera and flat-mounted on microscope slides. The labeled RGCs were counted in 4 groups (n = 10) in a masked fashion: normal and hypertensive eyes of animals receiving 5 mg/kg of KR-31378 and normal and hypertensive eyes of animals not receiving KR-31378. RGC were counted in 3 temporal regions (1 mm × 1 mm square) at a distance 1 mm from the optic disc, using a fluorescence microscope (Carl Zeiss, Jena, Germany).

Acute retinal ischemia and drug administration

Sprague Dawley rat (250 g, 8 weeks of age) were used for these experiments (n = 5). All animal procedures conformed to the guidelines of Catholic University of Korea and *Principles of Laboratory Animal Care* (NIH publication No. 85-23, revised 1985). Retinal ischemia was induced by high IOP in the anterior chamber. After anesthetization, a tube fitted with a 36-gauge needle connected to a pumping cylinder with pressure meter was connected into the cornea. This apparatus was used to elevate IOP above systolic blood pressure for 60 min (3). Conditions of retinal ischemia were confirmed by observed whitening of the retina. At the end of the experiments, the rats were sacrificed by lethal dose of anesthetic.

KR-31378 was administered in four different doses (5, 10, 30, and 50 mg/kg) by peritoneal injection, 10 min prior to induced ischemia. Glibenclamide (30 mg/kg; Sigma, St. Louis, MO, USA), which is an ATP-sensitive potassium-channel blocker, was peritoneally injected with or without KR-31378. For preparation of samples for Western blot analysis, retinas were collected 24 h post ischemia–reperfusion and stored at −70°C until protein extraction. For microscopy, retinas were collected 7 days post ischemia–reperfusion injury and fixed in 4% glutaraldehyde. The fixed retina were dehydrated and embedded in epon-mixture (Polyscience, Niles, IL, USA), sectioned at 1 μm by an ultramicrotome (Leica, Wetzlar, Germany), and stained with 1% toluidine blue. RGC were counted in five separate sections, from optic nerve to peripheral end in the temporal retina. Surviving RGCs were expressed as cell numbers for each group (mean ± S.E.M.).

Western blot analysis

Retinas were homogenized in lysis buffer [20 mM Tris (pH 7.4), 150 mM $NaCl_2$, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin]. After centrifugation, the supernatant was collected to determine total protein concentration by a BCA protein assay kit (Sigma). An amount of 20 μg protein from each sample was loaded on an SDS-polyacrylamide gel and separated by electrophoresis. Proteins were transferred to a nitrocellulose membrane (hybond-C; Amersham, Arlington Heights, IL, USA) at 350 mA for 1 h. The membrane was blocked with 5% skim milk and then incubated with monoclonal anti-GFAP and anti-α-tubulin antibodies (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After being washed in PBST (PBS containing 0.1% Tween 20), the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG for 1 h. The membranes were then washed with PBST buffer and visualized by applying chemiluminescent substrate (ECL, Amersham). Then the membranes were exposed to X-ray film (Fuji Film, Tokyo). The exposed films were analyzed by Image Master VDS software version 2.0 (Pharmacia Biotech, San Francisco, CA, USA).

Immunohistochemistry

At 24 h after reperfusion injury, animals were sacrificed, and the eyes were enucleated. The cornea and lens were removed, and the eye cups were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 30 min. The retina were dissected from the choroid and further fixed in the same fixative for 2 h at room temperature. After several washes in PB, small pieces of the retina were cut out from the region close to the optic nerve head and sectioned at 50-μm thickness by a vertical vibratome (Vibratome 1000C; Warner Instruments, Harnden, CT, USA). The sections were incubated in blocking solution [10% normal goat serum (NGS) in 0.01 M PBS (pH 7.4) containing 0.5% Triton X-100] for 1 h at room temperature. The sections were incubated with monoclonal anti-GFAP (1:500, Santa Cruz Biotechnology) in PBS containing 3% NGS and 0.5% Triton X-100 for 10 – 12 h at 4°C. After washes in PBS, the sections were incubated with HRP-conjugated anti-rabbit IgG for 2 h at room temperature. The immunoreactivity was visualized by using a 0.05% 3,3-diaminobenzidine (DAB) staining kit (Vector Laboratories, Burlingame, CA, USA). The light microscopy images were captured by an Olympus microscope equipped with a digital camera (Olympus, Tokyo)

Statistical analysis

The results were expressed as means ± S.E.M. The significance of differences was tested by the two-way analysis of variance (ANOVA) test.

Results

IOP reducing effect of KR-31378

When KR-31378 was administered orally in the experimental glaucoma model, the reducing effect of high IOP was observed (Fig. 1). In the non-treated group (hypertensive eyes without KR-31378 administration), the mean IOP was 31 ± 2 mmHg after cauterization and kept at the elevated level for 3 weeks. However, in the KR-31378–treated (30 mg/kg) group, the high IOP was decreased to normal levels (16 ± 1 mmHg) 2 weeks after the start of KR-31378 treatment (Fig. 1). In groups receiving 10 or 5 mg/kg KR-31378, the IOP declined to normal levels (16 mmHg) 18 days after the start of KR-31378 treatment. The IOP in the non-experimental left eye (normal eye) was not altered in animals receiving KR-31378.

The protective effect of KR-31378 against RGC cell death

In the normal retina, the RGC density was 2,461 cells/mm^2 (Fig. 2A), while the reduction of the RGC density (1,543 cells/mm^2) was observed in the hypertensive eye (Fig. 2C). We normalized the RGC density in the hypertensive eye by the RGC density in the normal retina. Percentage of surviving cells in the hypertensive eye was 63 ± 7% that of the normal retina (Fig. 2E). Administration of 5 mg/kg KR-31378 protected against the RGC death in the hypertensive eye (Fig. 2: D and E). After the KR-31378 administration,

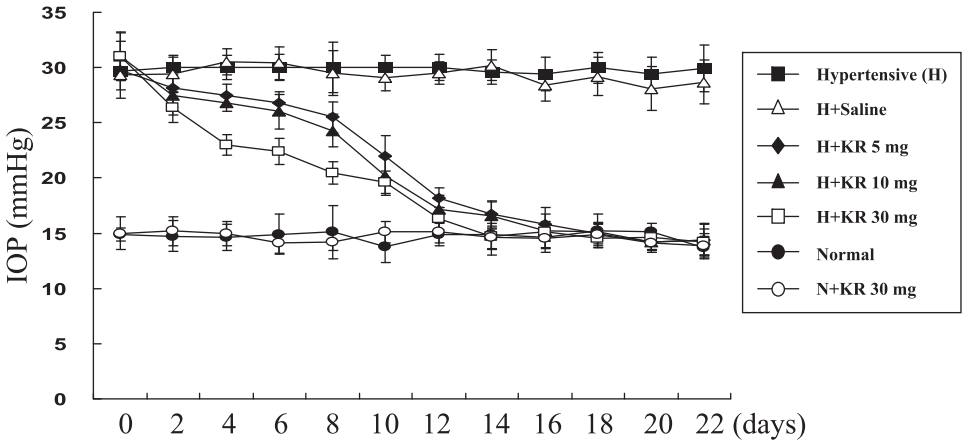

Fig. 1. Intraocular pressure (IOP)-reducing effect of KR-31378 in chronic ischemia model. KR-31378 was administrated orally in three different doses, two times per day (B.I.D). The doses are represented as 30 mg/kg (open squares), 10 mg/kg (filled triangles), and 5 mg/kg (filled diamonds). In all KR-31378–treated hypertensive eyes, the IOP was decreased to normal levels within 2 weeks after KR-31378 treatment. Hypertensive eyes without KR-treatment, filled squares; normotensive eyes without KR-treatment, filled circles; KR-31378 (30 mg/kg)-treated normotensive eyes, open circles; saline-treated hypertensive eyes, open triangles. Each IOP value is a mean ± S.E.M. (n = 10).

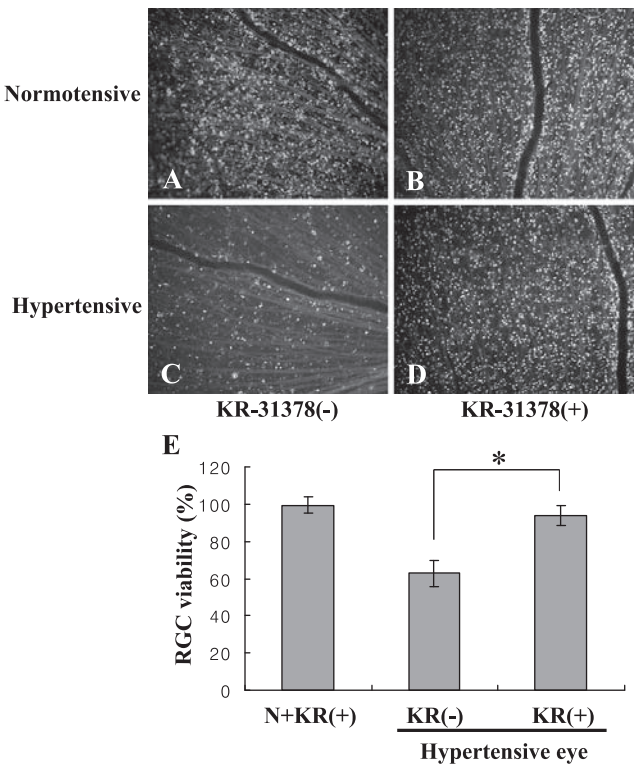

Fig. 2. Microphotograph of retinal flat mounts in the experiment using the chronic ischemia model. RGCs were labeled retrogradely by injection of 4Di-ASP10 into the superior colliculus. A and B are normotensive eyes, and C and D are hypertensive eyes. The RGC density in the KR-31378–treated hypertensive eyes group [KR-31378(+)] (D) is higher than that of non-treated hypertensive eyes group [KR-31378(−)] (C). Number of RGCs were counted in the temporal region (cells/mm^2) 6 weeks after KR-31378 (5 mg/kg) treatment (E). RGC viability is presented as the mean ± S.E.M. (n = 10). The number of RGCs in normotensive retinas was set at 100%. *P<0.01, by ANOVA.

survival ratio of RGC was 92 ± 5%. Administration of 5 mg/kg KR-31378 had no effect on the survival of RGC in the normal retina (Fig. 2: B and E).

Neuroprotective effect of KR-31378 against acute retinal ischemia

The number of RGCs was decreased in the non-treated retina after ischemia. However, in the KR-31378–treated groups, numerous RGCs remained in the ganglion cell layer (GCL) (Fig. 3C). The KR-31378–treated normal retina (Fig. 3B) was not different from non-treated normal retina (Fig. 3A). KR-31378 caused no morphological changes (Fig. 3B).

The protective effect of KR-31378 against acute retinal ischemia was observed at 30 or 50 mg/kg (51.7 ± 9 cells, 30 mg/kg; 57.3 ± 8 cells, 50 mg/kg). In ischemic retina, RGCs remained at 4.8 ± 5 cells in GCL (Fig. 4A). This protective effect was inhibited by glibenclamide (30 mg/kg, 18.4 ± 5 cells) (Fig. 4B). Also, the effect of KR-31378 was dose-dependently inhibited by glibenclamide (data not shown). A concentration of 30 mg/kg KR-31378 was used for the glibenclamide inhibition experiment because no significant difference was observed between KR-31378 treatment at 30 or 50 mg/kg.

To evaluate the effect of KR-31378 on the activation of Müller glial cells, we analyzed the expression of GFAP in ischemic retina by Western blot analysis (Fig. 5A) and immunohistochemistry (Fig. 5: B – I). In the normal retina, the immunoreactive band for GFAP was weak (Fig. 5A). At the immunohistochemical level, immunoreactivity for GFAP was detected in the nerve

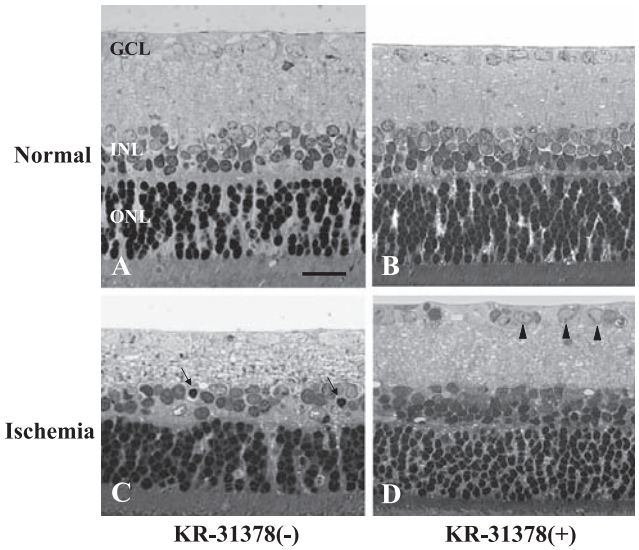

Fig. 3. Microphotographs of KR-31378–treated (B and D) and non-treated retina (A and C) in the acute ischemic model. Normal (A and B) and ischemic retina (C and D) are shown. In ischemic retina without KR-31378 treatment (C), RGC were not observed in the ganglion cell layer (GCL), and pyknotic cells (arrows) were observed in inner nuclear layer (INL). In KR-31378–treated ischemic retina (50 mg/kg, n = 5) (D), RGC (arrow heads) were observed in GCL. ONL, outer nuclear layer. Scale bar = 20 μm.

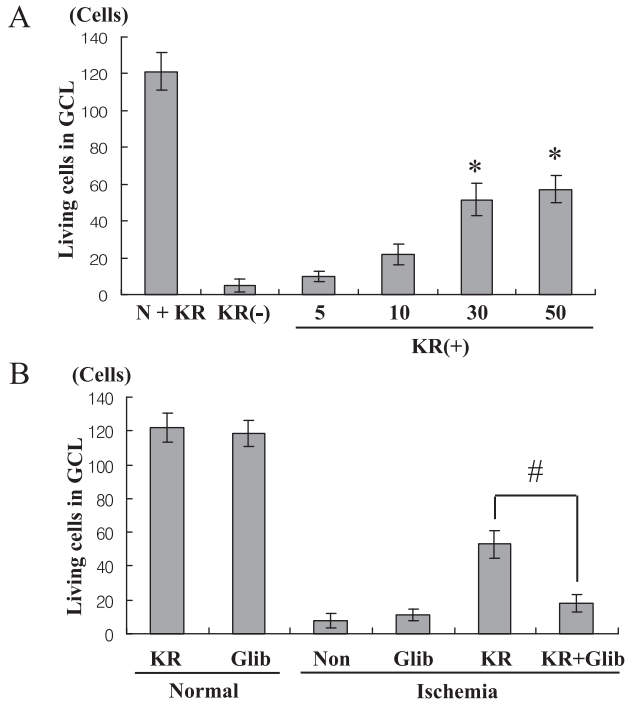

Fig. 4. The protective effect of KR-31378 against acute ischemia. A: KR-31378 (KR) was administered at four different doses, 5, 10, 30, and 50 mg/kg by intra-peritoneal injection [KR(+)] (n = 4, for each dose). KR-31378 dose-dependently exerted a protective effect against ganglion cell death. B: Neither KR-31378 (KR) nor glibenclamide (Glib) had any effect on normal retinal ganglion cells. However, the K_{ATP}-channel blocker glibenclamide (30 mg/kg, Glib) inhibited the protective effect of KR-31378 (n = 4, at each dose). The number of living cells in GCL is expressed as the mean ± S.E.M. $^{*}P<0.01$ vs. KR(−), $^{\#}P<0.001$. KR(−), ischemic retina without KR-31378 treatment; Non, ischemic retina without treatment of KR-31378 and glibenclamide.

fiber layer (NFL) (Fig. 5B). In the ischemic retina, the GFAP-immunoreactive band became strong (Fig. 5A) and immunoreactivity for GFAP was detected throughout the retina (Fig. 5F). In the ischemic retina that received an administration of KR-31378, GFAP expression was inhibited (Fig. 5H, weakly stained), and this inhibitory effect was blocked by glibenclamide (Fig. 5I, strong). In ischemic retina, glibenclamide did not inhibit GFAP expression (Fig. 5G). These results indicate that KR-31378 inhibits Müller glial cell activation after ischemic injury in the retina.

Discussion

We used two retinal ischemia models in this study. One experimental model has chronic ischemia characteristic of glaucoma. The other model has acute ischemia characterized by a temporary increase of IOP for 60 min, and this model offers the advantage of producing retinal neuronal degeneration in the absence of prolonged high IOP.

We observed the neuroprotective effect of KR-31378 in two retinal ischemia models by monitoring the degeneration of RGC. This neuroprotective effect was inhibited by glibenclamide, a K_{ATP}-channel blocker.

Previous studies have reported that K_{ATP} channels play an important role in enhancing retinal resistance against ischemic insult (10, 12, 14, 26). Retinal ischemic injury induces cell death of retinal neurons by excitotoxicity. In excitotoxic injury, increased glutamate causes continuous opening of NMDA or kainate channels, which destroys the retinal ion balance. The disturbed ionic environment is deleterious to retinal neurons and lead to cell death. Retinal Müller cells have K_{ATP} channels and can protect retinal neurons endogenously (8, 11).

KR-31378 was reported to be a potent K_{ATP}-channel opener and exhibits an antioxidant effect and anti-apoptotic activities. Mitochondrial K_{ATP}-channel opening is protective against ischemic brain injury in rats (23 – 25). In addition, the opening of potassium channels induces the reduction of K^+ ion levels in the cytoplasm, and this phenomenon is accompanied by an increase of the ischemic tolerance that mimics ischemic preconditioning (15). This is thought to act in the same way that a low level of K^+ ion inhibits or delays neuronal cell toxicity by Ca^{2+} influx in excitotoxicity. Therefore, KR-31378–induced K_{ATP}-channel opening can increase a

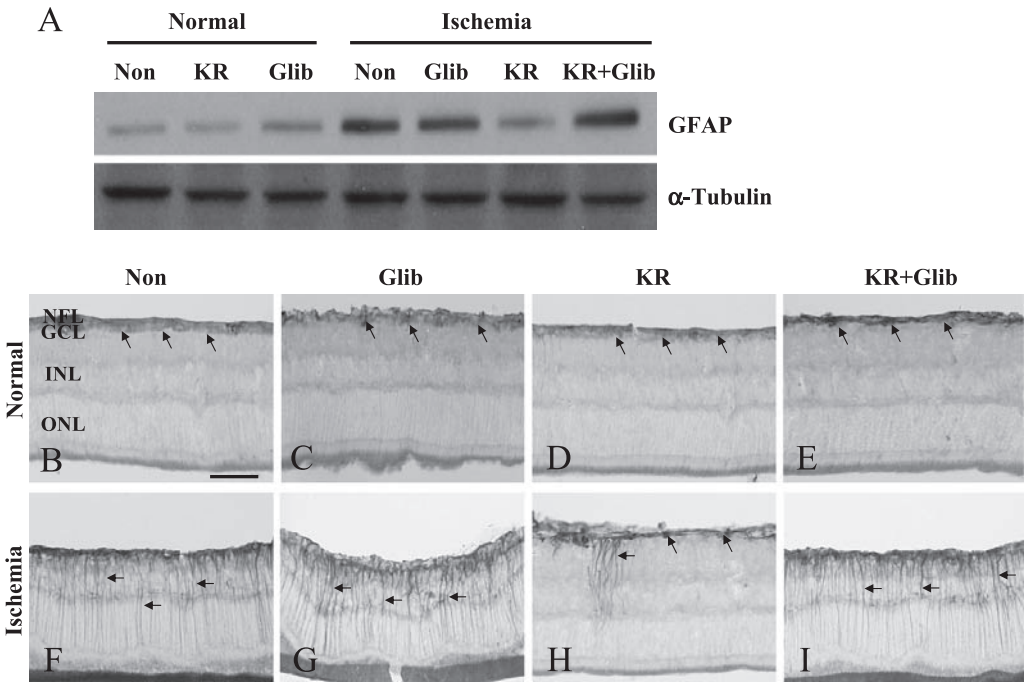

Fig. 5. The effect of KR-31378 on the expression of GFAP analyzed by Western blot analysis (A) and immunohistochemistry (B – I) in acute ischemic retina (n = 5, at each dose). In A, ischemic injury increased the level of GFAP expression. In ischemic retina, GFAP expression was inhibited by KR-31378 (30 mg/kg) treatment, and this effect was blocked by glibenclamide (30 mg/kg) co-treatment. GFAP expression was observed in normal retina and ischemic retina. GFAP expression was identified by black staining (arrows). B and F are non-treated retina; C and G are glibenclamide (30 mg/kg, Glib)-treated retina; D and H are KR-31378 (30 mg/kg, KR)-treated retina; E and I are co-treated retina. Results were representative of three independent experiments. NFL, nerve fiber layer. Scale bar = 50 μm.

resistance to ischemic injury and regulates ion balance during excitotoxicity. To confirm the retinal protective effect of KR-31378, we used glibenclamide, a K_{ATP}-channel blocker (15, 27), to test for the reversal of KR-31378 effects. Glibenclamide partially, but not completely, inhibited the protective effect of KR-31378 (Fig. 4). This partial inhibition may be due to the stringent specificity of glibenclamide as an ATP-sensitive potassium-channel blocker. KR-31378 can activate other potassium channels.

KR-31378 treatment inhibited GFAP expression following retinal ischemia (Fig. 5), and this effect was blocked by glibenclamide. Müller glial cell activation and increased GFAP expression are general phenomenon observed in ischemic-damaged neuronal tissues (28). Diazoxide, a K_{ATP}-channel opener, reduced the activation of glial cells in brain ischemia. Another potassium-channel opener, Iptakalim, increased glutamate uptake in cultured glial cells (16, 17). Reduction of extra-cellular glutamate concentration inhibits glutamate-induced neuronal toxicity in ischemia. Recent reports have shown that a defect in glutamate transporters induced reduction of glutathione levels in Müller cells and RGC degeneration (4). Since KR-31378 protects neurons from ischemia–reperfusion injury by attenuating glutathione loss (23), this drug may be suitable for use in the treatment of patients with high or normal IOP.

In the experimental glaucoma model, the number of RGCs in flat mounts of retinas obtained from KR-31378–treated animals was significantly higher than that in non-treated animals (Fig. 1). We also found that systemic administration of KR-31378 reduced IOP in the hypertensive eyes, while IOP in the control eye (uncauterized eye) was not reduced. In our experimental glaucoma model, assessment of the IOP-lowering effect was difficult because of the small change in IOP values; changes of 1 – 2 mmHg range were not easily measured in small eyes. However, the IOP-lowering effect in hypertensive eyes was easily measured. In this study, reduction of IOP by KR-31378 treatment increased the survival of RGC in the hypertensive eyes. Taken together, our results indicate that KR-31378 exhibits not only neuroprotective effects but also IOP reduction effects.

Current reports have demonstrated the role of potassium channels in glaucoma therapy. Potassium channels regulate the level of intracellular Ca^{2+} and thus control muscle relaxation and vasodilatation. Maxi-K channels and K_{ATP} channels are the predominate channels controlling relaxation of ciliary muscles and the trabecular

meshwork in eyes. Thus, an opening of these channels may increase uveal outflow (18 – 21).

These results suggest that KR-31378 works via dual mechanisms to exert two effects in high IOP–induced retinal ischemia: a neuroprotective effect and an IOP-reducing effect. To elucidate the biochemical mechanisms of KR-31378 action in glaucoma, further study is needed. In conclusion, we suggest that KR-31378 may be used to improve glaucoma therapies.

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