

# The Novel Rho Kinase (ROCK) Inhibitor K-115: A New Candidate Drug for Neuroprotective Treatment in Glaucoma

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**PURPOSE.** To investigate the effect of K-115, a novel Rho kinase (ROCK) inhibitor, on retinal ganglion cell (RGC) survival in an optic nerve crush (NC) model. Additionally, to determine the details of the mechanism of K-115's neuroprotective effect in vivo and in vitro.

**METHODS.** ROCK inhibitors, including K-115 and fasudil (1 mg/kg/d), or vehicle were administered orally to C57BL/6 mice. Retinal ganglion cell death was then induced with NC. Retinal ganglion cell survival was evaluated by counting surviving retrogradely labeled cells and measuring RGC marker expression with quantitative real-time polymerase chain reaction (qRT-PCR). Total oxidized lipid levels were assessed with a thiobarbituric acid-reactive substances (TBARS) assay. Reactive oxygen species (ROS) levels were assessed by co-labeling with CellROX and Fluorogold. Expression of the NADPH oxidase (Nox) family of genes was evaluated with qRT-PCR.

**RESULTS.** The survival of RGCs after NC was increased  $34 \pm 3\%$  with K-115, a significantly protective effect. Moreover, a similar effect was revealed by the qRT-PCR analysis of *Thy-1.2* and *Brn3a*, RGC markers. Levels of oxidized lipids and ROS also increased with time after NC. NC-induced oxidative stress, including oxidation of lipids and production of ROS, was significantly attenuated by K-115. Furthermore, expression of the Nox gene family, especially *Nox1*, which is involved in the NC-induced ROS production pathway, was dramatically reduced by K-115.

**CONCLUSIONS.** The results indicated that oral K-115 administration delayed RGC death. Although K-115 may be mediated through *Nox1* downregulation, we found that it did not suppress ROS production directly. Our findings show that K-115 has a potential use in neuroprotective treatment for glaucoma and other neurodegenerative diseases.

**Keywords:** oxidative stress, retinal ganglion cell, ROCK, glaucoma, neuroprotection, Nox

Glaucoma is well known as one of the world's major causes of secondary blindness,<sup>1</sup> and in Japan in particular, glaucoma is quickly becoming the most common cause of secondary blindness. Maintenance of low intraocular pressure (IOP) is the classic treatment for glaucoma and is the only therapy that has been shown to be effective in large-scale clinical studies. The primary method of reducing IOP is generally medication, mainly topical eye drops, although filtration surgery is also used. These are the only current treatments for glaucoma. Increased IOP is the most well-known risk factor for the progression of glaucoma, and IOP reduction is usually effective in slowing the progress of the disease. However, the majority of glaucoma patients in Asia are affected by normal tension glaucoma (NTG), and recent epidemiological studies have revealed that IOP reduction alone cannot prevent the progression of visual field loss in these patients.<sup>2,3</sup> In addition to reducing IOP, reduction of damage to retinal ganglion cells (RGCs) caused by IOP-independent risk factors such as mechanical stress on the axons in the lamina cribrosa might be useful for treating NTG.<sup>4</sup> Novel treatment strategies have therefore recently been explored, such as protecting RGCs or increasing retinal or choroidal blood flow. In particular, the neuroprotection of RGCs has drawn attention as a new approach

to glaucoma therapy because it is thought that the ultimate cause of vision loss in glaucoma is RGC apoptosis.<sup>5</sup>

Several potential mechanisms of RGC death in glaucoma have been hypothesized, including compromised blood flow in the optic nerve,<sup>6,7</sup> nitric oxide-induced injury to the optic nerve,<sup>8–10</sup> and glutamate excitotoxicity.<sup>11–13</sup> In addition to these primary mechanisms, other studies have provided evidence that oxidative stress contributes to the degeneration of RGCs in glaucoma.<sup>14–17</sup> However, the precise nature of the damage caused to RGCs by oxidative stress remains unclear. Moreover, treatments for oxidative stress in glaucoma patients have not been established.

Rho kinase (ROCK) is a serine/threonine (Ser/Thr) protein kinase and a key downstream effector of Rho.<sup>18,19</sup> ROCK controls multiple signaling pathways and many cellular processes such as cytoskeletal rearrangement and cell movement.<sup>20</sup> Thus, it has recently been suggested that the Rho/ROCK pathway is involved in a number of disorders. Indeed, abnormal activation of ROCK has been observed in diabetic nephropathy,<sup>21–26</sup> cardiovascular disease,<sup>19,24,27–31</sup> and central nervous system (CNS) diseases including Alzheimer's disease,<sup>32–34</sup> spinal cord injury,<sup>32,35–38</sup> stroke,<sup>39–46</sup> multiple sclerosis,<sup>32</sup> and glaucoma.<sup>47–56</sup> In particular, a recent study

reported that the protein level of RhoA increased in the optic nerve head of patients with primary open-angle glaucoma (POAG),<sup>57</sup> an effect that might lead to excessive activation of ROCK. Many studies using models such as hypertension, hyperlipidemia, and diabetes have demonstrated that ROCK activation caused elevated oxidative stress levels via NADPH oxidase (Nox), and that this was eliminated by oral administration of the ROCK inhibitor fasudil.<sup>22</sup>

ROCK inhibitors are thought to be one of the most promising candidates for the treatment of glaucoma. Previously, the targeting of small Rho GTPase has been shown to increase regeneration in models of optic nerve lesions.<sup>58</sup> Specifically, pharmacological inhibition of ROCK had a dose-dependent regenerative effect on RGCs after an optic nerve crush (NC) injury.<sup>59</sup> Moreover, selective ROCK inhibitors have also been shown to lower IOP in rabbits,<sup>48</sup> rats,<sup>60</sup> and monkeys.<sup>61</sup> This compound had a direct effect on the trabecular meshwork and the cells in Schlemm's canal. Recent research had provided a great deal of data on the multiple potential therapeutic uses of ROCK inhibitors in glaucoma, including both IOP maintenance<sup>48,53,55,62-65</sup> and RGC neuroprotection.<sup>66</sup>

K-115, an isoquinolinesulfonamide derivative, shows high selectivity for ROCK inhibition, especially ROCK 2. The 50% inhibitory concentration (IC<sub>50</sub>) of K-115 for ROCK 1, ROCK 2, PKA $\alpha$ , PKC, and CaMKII $\alpha$  was 0.051, 0.019, 2.1, 27, and 0.37  $\mu$ M, respectively.<sup>67</sup> In contrast, the IC<sub>50</sub> of other ROCK inhibitors such as Y-27632 and fasudil was 2 to 18 times higher than that of K-115. This high selectivity contributes to the safety profile of K-115 because different protein kinases have structurally similar active binding sites yet regulate diverse signaling pathways.<sup>32,68</sup> Indeed, phase 1 and 2 clinical trials have indicated that K-115 is a safe topical agent for IOP reduction over an 8-week course of treatment in healthy volunteers and patients with POAG.<sup>69,70</sup>

## METHODS

### Materials

Fluorogold (FG) was purchased from Fluorochrome (Denver, CO, USA). All chemicals used in this study's thiobarbituric acid-reactive substances (TBARS) assays were purchased from Wako Pure Chemicals (Osaka, Japan), except for the protease inhibitor cocktails, which were purchased from Sigma-Aldrich (Tokyo, Japan). K-115, a ROCK inhibitor, was kindly provided free of charge by Kowa Company, Ltd. (Nagoya, Japan). Fasudil was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

### Animals

Nine- to 12-week-old male C57BL/6 mice (SLC, Shizuoka, Japan) were used in this study. The animals in these experiments were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Guidelines for Animal Experiments of Tohoku University. All animal experiments were conducted with the approval of the Animal Research Committee, Graduate School of Medicine, Tohoku University. Every assay was conducted on a separate set of retinas.

### Retrograde Labeling of RGCs and Optic Nerve Surgery

To identify RGCs in the ganglion cell layer (GCL), retrograde labeling was performed 7 days before optic nerve surgery. Labeling was performed by injecting 1  $\mu$ L of 2% aqueous FG containing 1% dimethylsulfoxide (DMSO) into the superior

colliculus, using a Hamilton syringe with a 32-gauge needle. Seven days after retrograde labeling with FG, NC was performed as described previously.<sup>71,72</sup> Briefly, 15 minutes after administration of K-115 or fasudil, the optic nerve was crushed approximately 1 mm posterior to the eyeball without damage to the retinal blood supply. Beginning the day after surgery, K-115 or fasudil (1 mg/kg) was then administered orally once a day for 7 days.

### Quantitative Real-Time RT-PCR

The retinas were directly lysed in Qiagen RNeasy RLT Lysis buffer. Subsequent RNA extraction was performed with the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (50 ng) was reverse transcribed using a SuperScript III First Strand Synthesis kit (Life Technologies, Inc., MD, USA) to synthesize cDNA. Real-time quantitative RT-PCR was carried out with a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) using TaqMan probes (Life Technologies, Inc.). The catalog numbers of the predesigned TaqMan probes were as follows: Thy-1.2 (Mm00493681\_m1), Brn3a (Mm02343791\_m1), Nox1 (Mm00549170\_m1), Nox2 (Mm01287743\_m1), Nox3 (Mm01339132\_m1), Nox4 (Mm00479246\_m1), and GAPDH (Mm99999915\_g1). Relative gene expression levels were calculated using the delta-delta Ct method.

### TBARS Assay

The TBARS assay was carried out according to previously reported methods<sup>73,74</sup> with minor modifications. TBARS assays measure the total level of oxidized lipids based on the reaction of malondialdehyde (MDA), one of the end products of lipid peroxidation, with thiobarbituric acid (TBA).<sup>75</sup> Briefly, the retinal homogenate, in 1.15% KCl containing 1% protease inhibitor cocktail and 0.5 mM butylated hydroxytoluene (BHT), was added to a reaction mixture (0.81% SDS, 0.36% TBA, and 9% acetic acid) on ice. After heating the reaction mixture to 100°C for 1 hour, it was centrifuged at 20,000g for 10 minutes at 4°C. The supernatant was collected and its fluorescence was measured at 530 nm excitation and 550 nm emission. The results were normalized to protein concentration, which was determined with the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, MA, USA).

### In Vitro Lipid Peroxidation Inhibition Assay

Docosahexaenoic acid (DHA) was oxidized in a linoleic acid model system to measure antioxidant activity, following the method by Osawa and Namiki<sup>76</sup> with minor modifications. Briefly, K-115 was dissolved in PBS and added into a mixture of 5 mM DHA and an oxidizing agent (5 mM FeSO<sub>4</sub>/10 mM ascorbic acid). In a parallel experiment, the sample was replaced with a standard antioxidant, BHT, as a positive control. The mixed solution was induced at 37°C for 2 hours. The oxidized DHA solution (200  $\mu$ L) was added to 500  $\mu$ L of a reaction mixture (0.81% SDS, 0.36% TBA, and 9% acetic acid) on ice. After heating the reaction mixture to 100°C for 1 hour, it was centrifuged at 20,000g for 10 minutes at 4°C. The supernatant was collected and its fluorescence was measured at 530 nm excitation and 550 nm emission.

### In Situ Detection of ROS Production

FG-labeled mice were injected intravitreally with 1  $\mu$ L 50  $\mu$ M CellROX Green Reagent (Life Technologies, Inc.). A Hamilton syringe with a 32-gauge needle was used. Two hours after injection, the mice were perfused with ice-cold saline, followed by 4% paraformaldehyde (PFA). The eyes of the mice

were collected and fixed in 4% PFA for 1 hour on ice. Following fixation, the eyes were cryopreserved with increasing concentrations of sucrose and frozen in Tissue-Tek OCT compound (Sakura Finetech, Tokyo, Japan). For nuclear staining, 14- $\mu$ m-thick cryosections were incubated in propidium iodide (PI) solution for 10 minutes. FG- and CellROX-positive cells in the GCL were counted in complete retinal sections taken through the optic nerve. To avoid fluorescence bleed-through caused by FG, fluorescence microscopy was carried out without Vectashield mounting medium.

### Measurement of ROS Levels in the Retina

Two hours after the intravitreal injection of 1  $\mu$ L 50  $\mu$ M CellROX Green Reagent, the retinas were dissected in ice-cold Dulbecco's phosphate-buffered saline (DPBS) and frozen in liquid nitrogen. The retinas were then homogenized in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 1% protease inhibitor cocktail on ice, and centrifuged at 15,000g for 10 minutes at 4°C. The supernatant was collected and its fluorescence was measured at 485 nm excitation and 538 nm emission. The results were normalized to protein concentration, which was determined with BCA protein assay kit.

### Statistical Analysis

We used an unpaired *t*-test to evaluate statistical differences in the two samples. An ANOVA followed by Dunnett's test was used to compare the mean in the three groups. Data are presented as means  $\pm$  standard deviation. The level of statistical significance was set at  $P < 0.05$ .

## RESULTS

### K-115 Exerts a Neuroprotective Effect on RGCs After NC

There are several ROCK inhibitors that have been reported to attenuate neuronal cell death after optic nerve injury.<sup>49,59,77-79</sup> We used a mouse NC model to determine whether K-115, a novel ROCK inhibitor, exhibited the same neuroprotective effect on RGCs. The density of RGCs in the control and PBS treatment groups was  $3815 \pm 430$  RGCs/mm<sup>2</sup> and  $1730 \pm 196$  RGCs/mm<sup>2</sup>, respectively. Seven days after NC, the density of surviving RGCs in the K-115 and fasudil treatment groups decreased to  $3022 \pm 306$  RGCs/mm<sup>2</sup> and  $2846 \pm 89$  RGCs/mm<sup>2</sup>, respectively (Figs. 1B–J). We also performed qRT-PCR to evaluate the neuroprotective effects of K-115 and fasudil on RGCs. This revealed that after NC, the mRNA level of *Thy-1.2*, an early marker of RGC stress, fell by approximately 70, 50, and 40% in the PBS, K-115, and fasudil treatment groups, respectively (Fig. 1K). Similarly, the mRNA level of *Brn3a*, another marker of RGC, fell by approximately 90, 80, and 70%, respectively. Our results thus demonstrated a significantly increased RGC survival rate in the K-115- and fasudil-treated group, compared to the PBS-treated group. The neuroprotective effect of K-115 was transient, as it did not promote significant RGC protection at 14 or 28 days after NC (Supplementary Fig. S1).

### Inhibitory Effects of K-115 on Axonal Injury-Induced Lipid Peroxidation In Vivo

We previously found that 4-hydroxynonenal (4-HNE)- and 8-hydroxy-2'-deoxyguanosine (8-OHdG)-immunostained cells increased in the GCL after NC.<sup>72</sup> However, these markers do not

always reflect the overall oxidative status of the retina. Therefore, we measured the total level of oxidized lipids with a TBARS assay. We found that the level of TBARS in the retina increased with time after NC. As shown in Figure 2A, 4 and 7 days after NC, oxidized lipids had increased significantly in comparison with the non-NC group ( $2.0 \pm 0.6$  vs.  $3.4 \pm 1.1$  and  $4.8 \pm 0.7$  nmol/mg protein). We next investigated whether K-115 inhibits lipid peroxidation in the retina after NC. We found that administration of 1 mg/kg/d of K-115, which qRT-PCR analysis of RGC markers such as *Thy-1.2* and *Brn3a* revealed was an effective concentration (data not shown), significantly attenuated the oxidation of lipids in the retina after NC (Fig. 2B). This result suggests that K-115 can inhibit the oxidative stress induced by axonal injury.

### Antioxidant Effects of K-115 on the Free Radical-Mediated Oxidative System

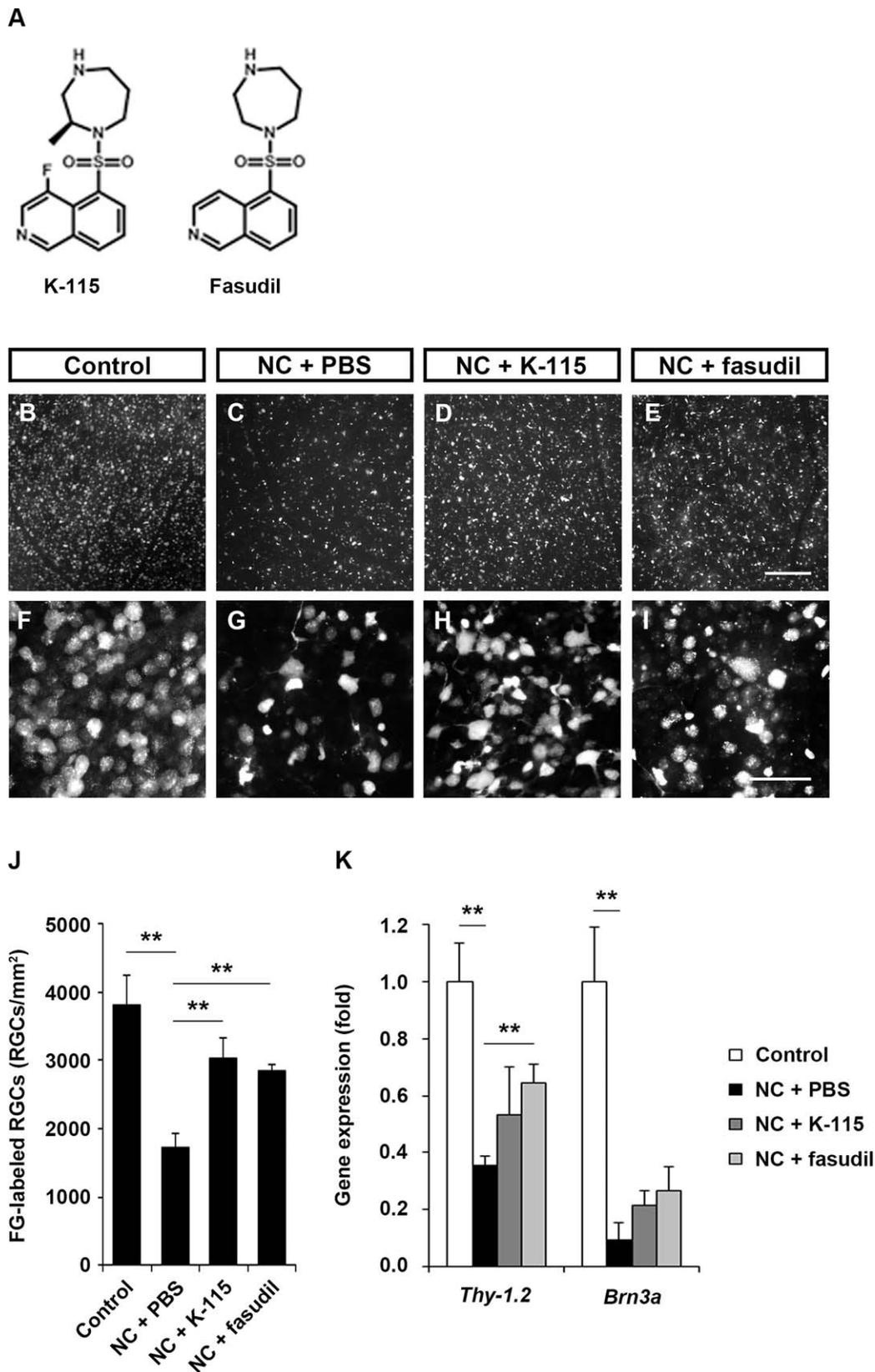
To further determine the inhibitory effect of K-115 on lipid peroxidation after NC, we measured the TBARS level induced by free radical-mediated oxidative stress. Since it is well known that DHA is the major polyunsaturated fatty acid (PUFA) in the retina,<sup>80</sup> we assessed the oxidative level of DHA in an in vitro system. Our in vivo system clearly indicated that K-115 had an inhibitory effect on NC-induced lipid peroxidation. However, as the functional mechanism behind this effect remained unclear, we tried to determine if K-115 functions directly as an antioxidant. Butylated hydroxytoluene, a well-known synthetic antioxidant, efficiently delayed lipid peroxidation in comparison to an untreated group, whereas the delay in peroxidation after the administration of K-115 was significantly lower (Fig. 3). This indicated that K-115 did not, in fact, act as an antioxidant reagent in this system.

### Identification of ROS-Generating Cells in the GCL After NC

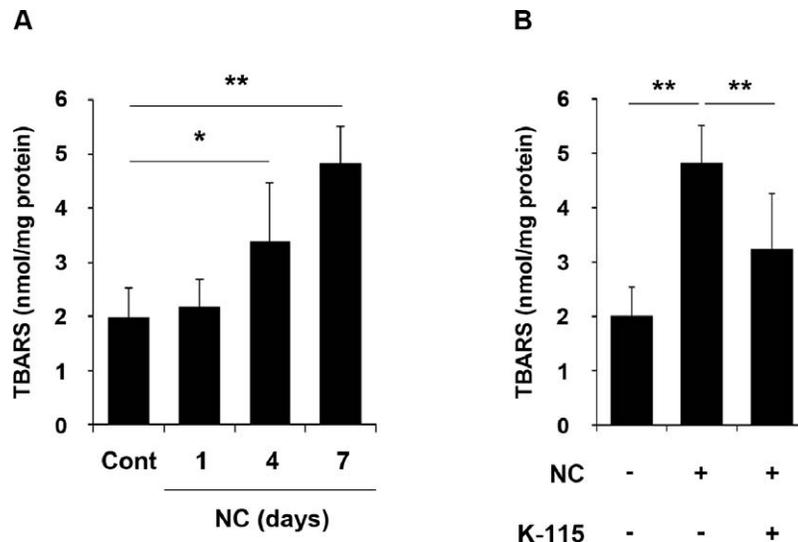
Previously, our group found oxidative stress markers such as 8-OHdG and 4-HNE in the GCL after NC,<sup>72</sup> clearly indicating that NC induces oxidative stress. Since ROS, including free radicals such as superoxide anions and hydroxyl radicals, are one of the main contributors to oxidative stress, we attempted to identify the major source of ROS production in the GCL by performing double labeling with the retrograde tracers FG and CellROX, since these accumulate in the mitochondria and nucleus. As shown in Figure 4A, the cells in the GCL that were positive for the CellROX fluorescence signal were mostly RGCs. Interestingly, however, some cells in the inner nuclear layer (INL) also produced ROS 4 days after NC. The percentages of FG/CellROX double-positive cells among the GCL cells 1, 4, and 7 days after NC were 58, 88, and 73%, respectively (Fig. 4B). The percentage of non-RGC cells positive for CellROX cells in the GCL reached a maximum of 11% on day 4. These results indicate that production of ROS after NC occurs mainly in RGCs.

### K-115 Suppressed the Time-Dependent Production of ROS in RGCs After NC Injury

As shown in Figures 4A and 4B, CellROX labeling identified the location of ROS production, indicating that oxidative stress is mainly induced in RGCs after NC. Furthermore, as shown in Figure 1, K-115 dramatically altered the RGC death rate after NC. This prompted an investigation of K-115's role in suppressing ROS production in RGCs using an in vivo model, by first inducing ROS production with NC, and then assessing the level of CellROX fluorescence. We found that after NC, the percentage of FG/CellROX double-positive cells gradually



**FIGURE 1.** K-115 and fasudil exerted a neuroprotective effect on RGCs after NC. **(A)** Chemical structures of K-115 and fasudil. **(B–I)** Representative images of retrogradely labeled RGCs. **(B–E)** Higher-magnification versions of the upper panels. *Scale bars:* 200  $\mu\text{m}$  **(B–E)** 50  $\mu\text{m}$  **(F–I)**. **(J)** Oral administration of K-115 or fasudil (1 mg/kg daily) for 7 days significantly delayed cell death in post-NC RGCs ( $n = 6$  in each group). **(K)** Treatment with K-115 or fasudil also delayed a reduction in mRNA of *Thy-1.2* and *Brn3a*, RGC markers. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal standard (\*\* $P < 0.01$ ; error bars, SD;  $n = 4$  in each group).



**FIGURE 2.** Inhibitory effects of K-115 on axonal injury-induced lipid peroxidation in vivo. (A) After NC, the level of lipid peroxidation increased in a time-dependent manner in the retina compared with the nontreated control group ("Cont"). (B) Daily administration of K-115 for 7 days significantly inhibited lipid peroxidation in mice after NC (\* $P < 0.05$ , \*\* $P < 0.01$ ; error bars, SD;  $n = 6$  in each group).

increased, reaching a maximum of 94% on day 4 before decreasing to 78% on day 7 (Figs. 5A, 5B). With K-115 treatment, although the percentage of CellROX-positive RGCs decreased in a time-dependent manner for 7 days, at all time points the percentage of double-positive cells was at least 40% lower than with PBS treatment. We also found that CellROX fluorescence intensity in the retinal lysate was significantly increased after NC, and that this elevated intensity was almost completely suppressed by administration of K-115 (Fig. 5C). These results indicate that K-115 treatment, rather than inhibiting downstream ROS production after NC, such as lipid peroxidation, inhibits ROS production itself through an indirect mechanism.

### Involvement of the Nox Family in NC-Induced ROS Production

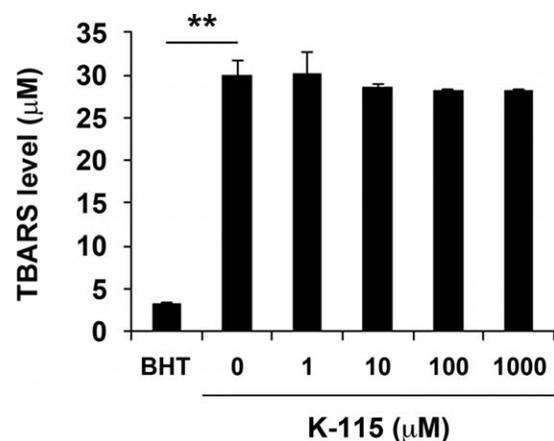
As previous reports indicated that the Rho/ROCK pathway is involved in inducing the Nox family,<sup>22,81,82</sup> we also performed an investigation of the involvement of this family with ROS production after NC. After NC, the mRNA levels of *Nox1*, *Nox2*, and *Nox4* in the PBS treatment group increased 5.6-, 3.2-, and 5.7-fold relative to the control group (Fig. 6A). While K-115 had an inhibitory effect on NC-induced up-regulation of *Nox1*, it had no effect on *Nox2* and *Nox4*. We next considered the direct involvement of the Nox family in NC-induced ROS production. We found that within 5 minutes of treatment with the Nox inhibitor VAS2870 after NC, CellROX fluorescence was almost completely eliminated. This suggests that almost all ROS production is derived from *Nox1*.

### DISCUSSION

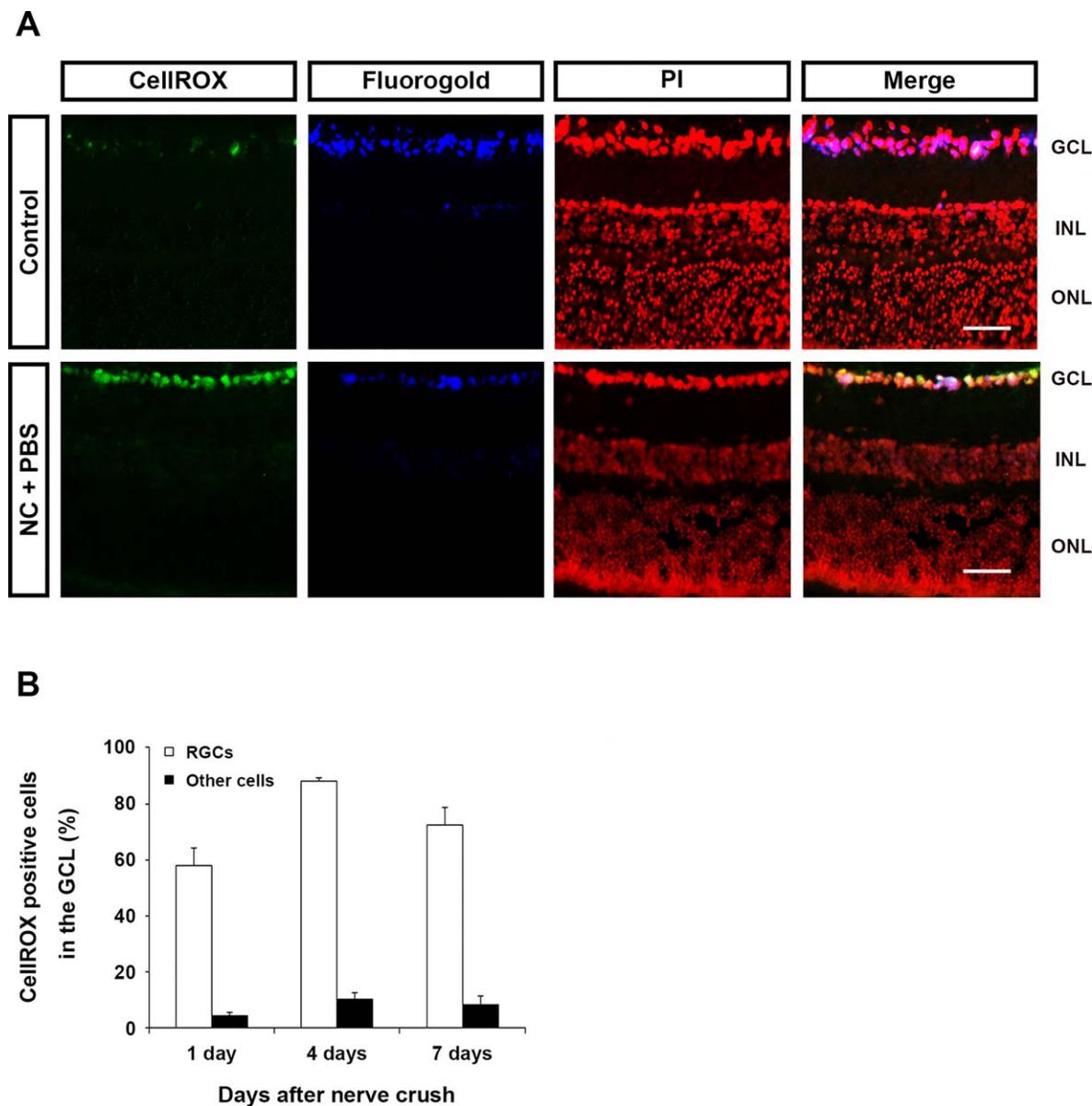
The results of the present study strongly suggest that K-115, a ROCK inhibitor, can prolong RGC cell survival by suppressing oxidative stress through pathways involving the Nox family (Fig. 7).

Both our present study and previous studies by others have shown that oxidative stress is involved in RGC death after axonal injury.<sup>17,72,83-89</sup> In order to evaluate the efficacy of K-115, we compared the density of FG-labeled RGCs in mice treated with either PBS or ROCK inhibitors such as K-115 and

fasudil 7 days after NC, in addition to the qRT-PCR analysis of RGC markers. We found that the neuroprotective effects of K-115 and fasudil after NC were similar, but that the specificity of the effect of K-115 on ROCK was 2 to 18 times higher than that of fasudil. This might have been related to our finding that the concentration-dependent neuroprotective effect of K-115 and fasudil against active ROCK in the retina after NC reached a plateau at 1 mg/kg/d. We therefore speculate that both ROCK inhibitors have a similar protective effect against axonal injury. Additionally, we found that K-115 dramatically suppressed oxidative stress, including ROS production by the RGCs themselves. Although the precise mechanism by which K-115 suppresses ROS production after NC has not yet been adequately determined, our present results strongly indicate that K-115 does not directly function as an antioxidant (Fig. 3). Moreover, we confirmed that expression of *Nox1*, which is strongly related to ROS production after NC, decreased with K-115 treatment. Based on previous findings that fasudil, a compound whose structure is similar to K-115, had an indirect antioxidant effect in various disease models including hyper-



**FIGURE 3.** Antioxidative effects of K-115 on the free radical-mediated oxidative system. The synthetic antioxidant BHT (50 mM) delayed lipid peroxidation efficiently, but K-115 (1–1000 μM) did not show an antioxidative effect (\*\* $P < 0.01$ ; error bars, SD;  $n = 3$  in each group).



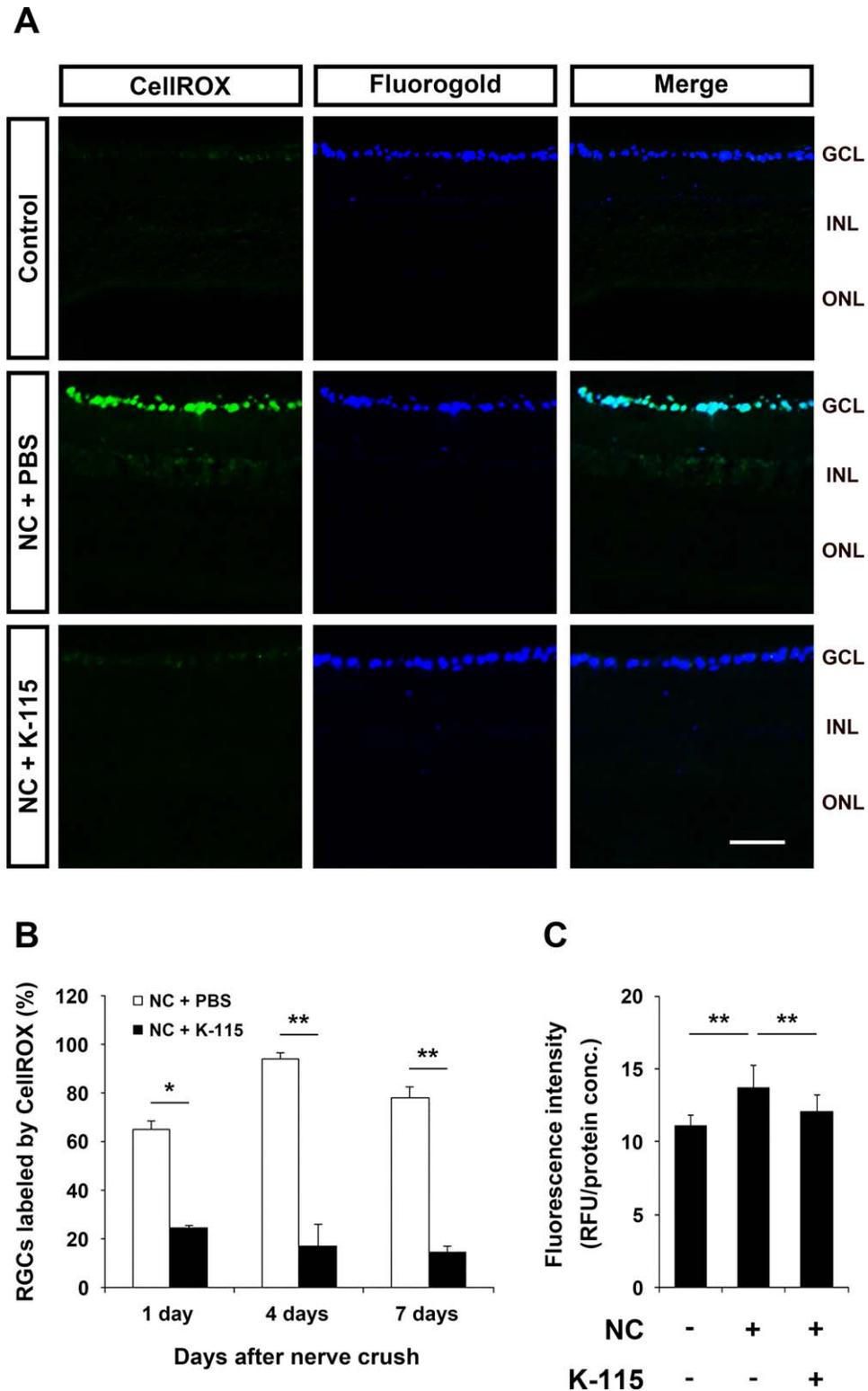
**FIGURE 4.** Identification of ROS-generating cells in the GCL after NC injury. **(A)** Representative fluorescence images of frozen sections showing ROS-producing cells (shown in *green*) in the GCL on day 4 after NC. *Scale bars:* 50  $\mu$ m. **(B)** Quantification of the number of FG-positive (shown in *blue*) or -negative cells among the CellROX-labeled cells on days 1, 4, and 7 after NC injury (day 1  $n = 4$ , days 4 and 7  $n = 6$ ). Cell counting results are shown as the ratio of FG- and CellROX-positive cells (i.e., RGCs) or FG-negative and CellROX-positive cells (i.e., other cells) to the number of PI-positive cells (*red*) in each section.

cholesterolemia,<sup>90</sup> diabetes,<sup>91</sup> and ischemia,<sup>92</sup> it is possible that K-115 also had a similar antioxidant effect in our NC model.

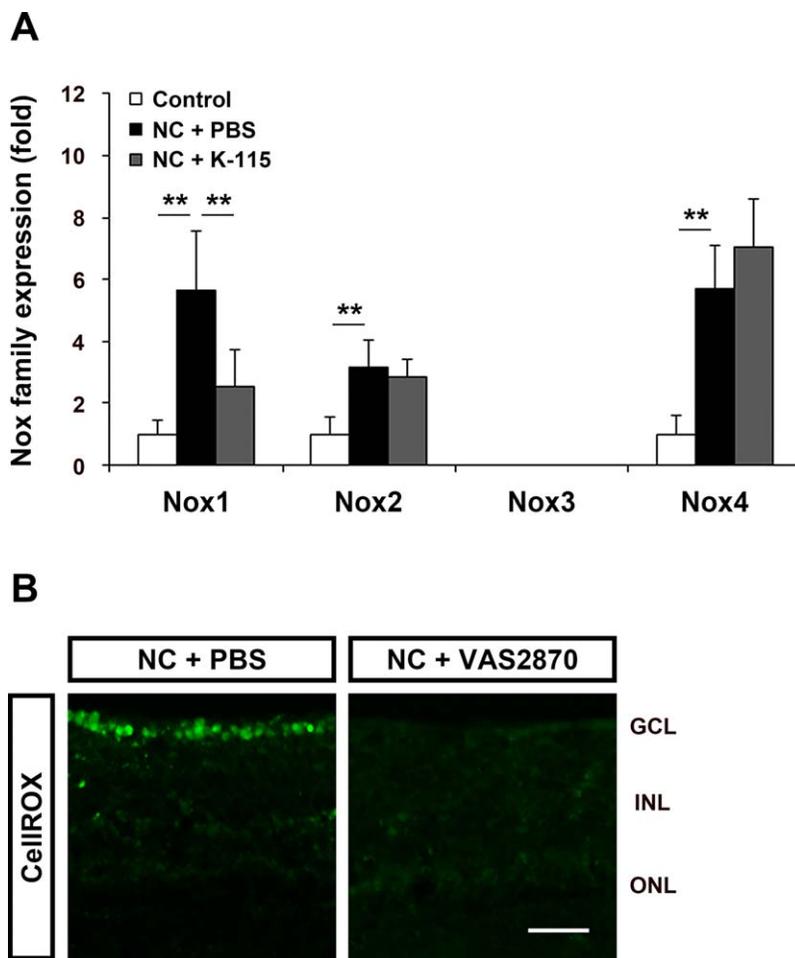
Oxidative stress is implicated in neuronal cell death in many neurodegenerative diseases, such as Alzheimer's disease,<sup>34</sup> amyotrophic lateral sclerosis,<sup>93</sup> and Parkinson's disease.<sup>94,95</sup> In glaucoma it has been reported that increases in oxidative stress markers can be found in a patient's aqueous humor and plasma.<sup>86,87</sup> Our research to date, using an experimental glaucoma model, strongly suggests that glaucoma should also be considered as a chronic neurodegenerative disease associated with oxidative stress. One widely accepted measurement of oxidative stress is the TBARS assay. Lipid peroxides, unstable indicators of oxidative stress in cells, decompose to form more complex and reactive compounds. Measurements of these end products indicate the level of oxidative damage. Our study demonstrated that the TBARS level in the entire retina

increased with time after NC, and was highest on the seventh day. However, as indicated in our previous report, oxidative stress appears to be higher in RGCs than in any other layer of the retina.<sup>72</sup> Therefore, we suggest that oxidative stress-induced RGC death directly affects the survival of RGCs after NC. To protect RGCs from oxidative stress, we examined the effects of the ROCK inhibitor K-115, and found that it could inhibit increases in the TBARS level. In other words, K-115 can suppress oxidative stress after NC.

ROCK inhibitors have been widely used as treatments for various neurological disorders, including spinal cord injuries,<sup>32,35-38</sup> stroke,<sup>39-46</sup> multiple sclerosis,<sup>32</sup> and Alzheimer's disease.<sup>32-34</sup> It has previously been reported that targeting small Rho GTPase has a positive dose-dependent effect on the regeneration of RGCs after injuries such as NC.<sup>49,59,77-79</sup> In the axonal injury model, it has been reported that ROCK activity



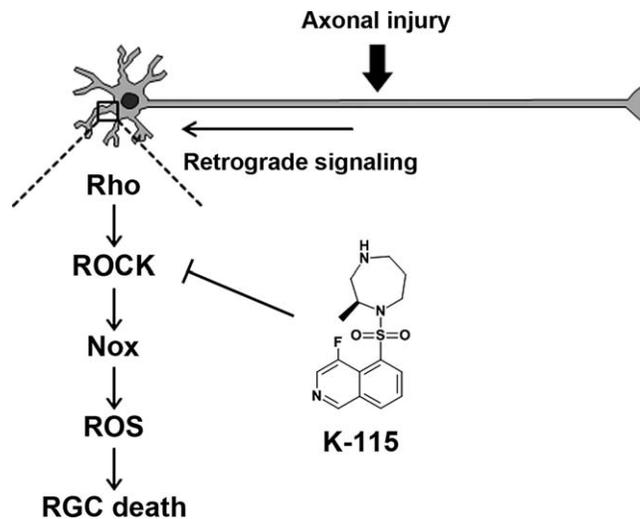
**FIGURE 5.** K-115 suppressed the time-dependent production of ROS in RGCs. (A) Representative fluorescence images of frozen sections confirming ROS production (green) in FG-labeled RGCs (blue) on day 4 after NC. K-115 treatment attenuated the NC-induced increase in CellIROX fluorescence in RGCs. Scale bar: 100  $\mu$ m. (B) Quantification of the number of CellIROX positive cells among FG-labeled RGCs on days 1, 4, and 7 after NC injury. K-115 significantly reduced the number of CellIROX-labeled cells among the retrogradely labeled RGCs at each time point ( $*P < 0.05$ ,  $**P < 0.01$ ; error bars, SD; PBS group: day 1  $n = 4$ , days 4 and 7  $n = 6$ ; K-115 group: day 1, 4  $n = 6$ , day 7  $n = 5$ ). (C) Measurement of ROS levels using a fluorophotometer. Fluorescence intensity (RFU) was normalized to protein concentration (mg/mL), which was determined with a BCA protein assay kit ( $*P < 0.05$ ,  $**P < 0.01$ ; error bars, SD;  $n = 6$  in each group).



**FIGURE 6.** Involvement of the Nox family in NC-induced ROS production. (A) Treatment with K-115 suppressed induction of the Nox family, *Nox1-4* mRNA, at day 4 after NC. *GAPDH* was used as an internal standard (\*\**P* < 0.01; error bars, SD; *n* = 8 in each group). (B) Representative fluorescence images of frozen sections showing that axonal injury-induced ROS production was greatly reduced by treatment with the Nox inhibitor VAS2870 (10 pmol/eye) at day 4. VAS2870 was intravitreally injected within 5 minutes of NC (*n* = 4 in each group). Scale bar: 100  $\mu$ m.

increased in the RGC layer, reaching a maximum on the fourth day after injury.<sup>59</sup>

Moreover, the increase in ROCK activity was not observed in other retinal layers, implying that this response to axonal lesions is specific to RGCs. However, we had difficulty at first finding evidence directly indicating which oxidative stress pathway was suppressed by the ROCK inhibitor K-115. Figure 3 shows our assessment of the ability of ROCK inhibitors to directly suppress oxidative stress. The antioxidant effect of K-115 was significantly lower than that of BHT. This suggests that K-115 did not act as an antioxidant reagent in this system, and led us to believe that ROCK inhibitors increase antioxidant activity through another mechanism. Looking to test this hypothesis, we assessed the gene expression pattern after NC of the Nox family, including *Nox1*, 2, 3, and 4, with K-115 treatment. Previously, it was reported that the Nox family is expressed downstream of ROCK activity,<sup>22,81,82,96</sup> and indeed, we did find that the expression of *Nox1*, 2, and 4 increased after NC, particularly *Nox1* and 2 in the RGCs. Previous findings that *Nox1*, 2, and 4 were expressed in surviving RGCs, and that RGCs had a significantly higher level of *Nox1* than other members of the Nox family,<sup>96</sup> support our results. It has also been reported that *Nox2* is particularly expressed in the microglial cells.<sup>97,98</sup> The final results of the experiments reported here showed that only *Nox1* was suppressed after NC with K-115 treatment. This decrease in *Nox1* expression after K-115 treatment supported our hypo-



**FIGURE 7.** K-115 inhibited oxidative stress via the Rho/ROCK pathway in RGCs. Axonal injury resulted in Nox-mediated ROS production via the Rho/ROCK pathway, which was attenuated by K-115.

esis. Indeed, Nox1 was found to play a critical role in ischemia-induced oxidative stress and RGC death in experiments using the Nox inhibitor VAS2870.<sup>96</sup> Furthermore, since K-115 also significantly inhibits RGC death in ischemia-reperfusion models (Mizuno K, et al. *IOVS* 2007;48:ARVO E-Abstract 4805), this protective effect might have a similar mechanism that modulates Nox family expression.

It is widely accepted that mitochondrial ROS cause oxidative damage to nuclear DNA. Mitochondrial-derived death signaling has previously been reported to be an important pathway for RGC death induced by axonal damage.<sup>99,100</sup> Mitochondria are also known to be abundant in the optic nerve.<sup>101</sup> Previously, we detected ROS in the mitochondria of RGCs, suggesting that axonal damage affects mitochondrial function, which in turn triggers RGC death.<sup>72</sup> In the present study, we have confirmed that oxidative stress is also involved in NC-induced RGC death. In this study we present evidence, using CellROX staining, that NC-induced apoptosis in RGCs produced high amounts of ROS. Indeed, the reduction in the number of ROS-producing cells with K-115 treatment was confirmed by both counting the cells (Fig. 5B) and by a fluorophotometric analysis (Fig. 5C). However, the difference between the PBS and K-115 treatment groups, shown in Figure 5C, were smaller than in the groups shown in Figure 5B. The different results obtained from these two analyses raise the possibility that our fluorophotometric evaluation of the suppressive effect of K-115 on ROS production after NC may have been affected by the difficulty of measuring the fluorescence intensity of retinal lysates. ROS are generated by the process of isolating the retina itself, even if the retinas are immediately dissected in ice-cold DPBS. Therefore, we consider that counting the number of cells was the most suitable method to evaluate K-115's effect on ROS production. The metabolic processes involving the mitochondrial electron transport chain are known to contribute to the formation of harmful ROS. Furthermore, our results indicated that K-115 significantly suppressed NC-induced oxidative stress by inhibiting ROS production in RGCs.

Our results thus strongly suggest that the prevention of oxidative stress in the mitochondria or nucleus should be regarded as candidates for the treatment of glaucoma. Furthermore, we believe that we have shown that suppression of Rho activity also has the potential to be a new neuroprotective treatment for glaucoma, particularly NTG (the main type of glaucoma in Asian countries).<sup>102</sup>

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