Axonal Protection by Ripasudil, a Rho Kinase Inhibitor, via Modulating Autophagy in TNF-Induced Optic Nerve Degeneration

Yasushi Kitaoka,1,2 Kana Sase,1 Chihiro Tsukahara,1,2 Kaori Kojima,1 Akira Shiono,1 Jiro Kogo,1 Naoto Tokuda,1 and Hitoshi Takagi1

1Department of Ophthalmology, St. Marianna University School of Medicine, Kawasaki, Kanagawa, Japan
2Department of Molecular Neuroscience, St. Marianna University Graduate School of Medicine, Kawasaki, Kanagawa, Japan

Correspondence: Yasushi Kitaoka, Department of Ophthalmology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan; kitaoka@marianna-u.ac.jp.

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PURPOSE. The Rho kinase inhibitor ripasudil decreases intraocular pressure, although its role in optic nerve axonal damage should be clarified. We therefore investigated whether ripasudil modulates TNF-induced axonal loss and affects autophagy machinery after the induction of optic nerve degeneration.

METHODS. Rats were given intravitreal injection of TNF, concomitant injection of ripasudil hydrochloride hydrate and TNF, or ripasudil alone. Axon numbers were counted to evaluate the effects of ripasudil against axon loss. Immunoblot analysis was performed to examine p62 as well as LC3-II expression in optic nerves Electron microscopy was used to determine autophagosomes in axons and glia. Immunogold labeling was performed to evaluate autophagosomes in axons.

RESULTS. Ripasudil injected intravitreally resulted in significant neuroprotection against TNF-induced axon loss. Intravitreal TNF injection upregulated p62 in the optic nerve, but ripasudil completely inhibited this increment. The ripasudil alone injection diminished p62 and enhanced LC3-II protein levels significantly compared with baseline. Ripasudil-induced upregulation of LC3-II was seen after TNF injection, and immunohistochemical analysis revealed that LC3 colocalized in nerve fibers. Electron microscopic analysis revealed that autophagosomes were present in axons and glia, although autophagosome numbers increased significantly after ripasudil injection only in axons.

CONCLUSIONS. These results suggest that ripasudil-enhanced intra-axonal autophagy is at least partly involved in axonal protection.

Keywords: p62, LC 3-II, ripasudil, autophagy, tumor necrosis factor, immunogold

The Rho kinase (ROCK) inhibitor ripasudil hydrochloride hydrate lowers intraocular pressure (IOP). The mechanism of the IOP-reducing effects of several ROCK inhibitors has been documented. For example, Y-27632, a ROCK inhibitor, may alter the behavior of trabecular meshwork (TM) cells, resulting in IOP reduction.4 Conventional aqueous humor outflow is significantly increased by Y-39983, another ROCK inhibitor, thus reducing IOP.5 One review concluded that ROCK inhibitors likely increase conventional aqueous humor outflow, accompanied by reorganization of the TM cells, and increased giant vacuoles in Schlemm’s canal endothelial cells.3 A recent study has shown that ripasudil increases outflow facility while modulating TM cell behavior and Schlemm’s canal endothelial cell permeability and disrupting tight junctions.4 Therefore, although the mechanism by which ripasudil lowers IOP is fairly well understood, its role in optic nerve axons must still be clarified.

Various studies demonstrated the crucial roles autophagy plays in glaucomatous damage.5–7 Some recent studies have suggested a detrimental role of p62 (SQSTM1) with regulation of autophagy in certain conditions in neurodegeneration.8–10 We previously reported upregulated p62 in the optic nerve in two different models such as a hypertensive glaucoma model and TNF-induced axon damage model.11–12 Microtubule-associated protein light chain 3 (LC3) is distributed in autophagosomes and LC3-II correlates with autophagosomes numbers.13 Enhanced autophagy in optic nerves may lead to diminished p62 levels and upregulated LC3-II protein levels.5,11,15 Because TNF is associated with certain types of glaucoma,16–19 the TNF-mediated axon damage model may be used to elucidate the mechanism by which axons are degenerated in retinal ganglion cells (RGCs).20 We undertook the present study in an attempt to confirm that ripasudil alters axonal loss caused by TNF and whether it modifies the optic nerve autophagy machinery.

MATERIALS AND METHODS

Animals

We used 8-week-old male Wistar rats. We used 19, 56, 6, and 3 rats were used for the axon morphometric analysis, immunoblot analysis, immunohistochemical analysis, and immunogold labeling studies, respectively. Three eyes per experimental condition from the axon counting study were used for electron microscopic analysis. Experiments were approved by the ethics...
committee for Animal Experiments of St. Marianna University School of Medicine and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were maintained under environmentally controlled conditions (12-hour light/12-hour dark).

**TNF Injection Model**

Intravitreal injection was carried out as described previously. Anesthetized rats were administered by intravitreal injection of 10 ng TNF (Sigma-Aldrich Corp., St. Louis, MO, USA) to the right eye and PBS to the contralateral left eye. For the ripasudil treatment, concomitant injection of 2, 20, or 200 pmol of ripasudil hydrochloride hydrate (Kowa Co., Ltd., Nagoya, Japan) and 10 ng of TNF was performed intravitreally; ripasudil 200 pmol alone was also administered intravitreally. One or 2 weeks following injection, the rats were euthanatized with overdose sodium pentobarbital, and the optic nerves were then collected.

**Immunoblot Analysis**

As described previously, 1, 30 rats or 2 weeks (26 rats) following injection, optic nerve samples were obtained, homogenized, and centrifuged. One sample contained two pooled optic nerve specimens. Each protein sample (5 µg/lane) was loaded and transferred to polyvinylidene fluoride membranes. After blocking, they were reacted with anti-p62 antibody (1:200); anti-LC3 antibody (1:200); or anti-β-actin antibody (1:500). After washing, the membranes were reacted with horseradish-labeled secondary antibodies (1:500). Antibody (1:100) or anti-neurofilament-L antibody (1:100). After washing, the sections were reacted with rhodamine-labeled or FITC-labeled secondary antibodies (1:1000). After several washing, signals were detected by an enhanced chemiluminescence system.

**Immunohistochemical Analysis**

One (3 rats) or 2 weeks (3 rats) following intravitreal injection of PBS or ripasudil, samples were put into 10% neutral-buffered formalin. After paraffin sections were made, sections were incubated with anti-LC3 antibody (1:100) or anti-neurofilament-L antibody (1:100). After washing, the sections were reacted with rhodamine-labeled or FITC-labeled secondary antibodies (1:100) in the dark. After several washing, the sections were mounted on slides in 4 ′,6-diamidino-2-phenylindole-containing medium (Vectashield; Vector Laboratories, Burlingame, CA, USA). Bovine serum was placed as a negative control.

**Morphometry of Axon in Optic Nerves**

Optic nerve axons were analyzed morphometrically as described previously using samples from 19 rats. Plastic sections were made starting from 1 mm behind the eye ball and stained with 1% paraphenylene-diamine (Sigma-Aldrich Corp.) solution. Axon numbers were quantified in five separate areas (center and periphery in quadrant; 1446.5 µm² each; totaling 7232.3 µm² per optic nerve) using image-processing software. The axon numbers in each sample were averaged and presented as the number per mm².

**Electron Microscopy**

Ultrathin sections (100 nm) were made from the acrylic resin blocks, incubated with saturated uranyl acetate, and examined by using a transmission electron microscope (TEM; JEOL, Tokyo, Japan). Autophagosome numbers inside axons and in glia were determined as the sum in 10 different areas of 10.24 µm² each (totaling 102.4 µm² per optic nerve) from each sample. The analysis was performed in three eyes per experimental condition.

**Immunogold Labeling**

Three rats were used for the immunogold labeling study. Two weeks following injection of PBS or ripasudil, optic nerves were incubated in a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde for 4 hours. After infiltration, optic nerves were embedded in LR-white resin (London Resin Company, Berkshire, UK) in gelatin. Ultrathin sections were made and mounted on nickel grids (300 mesh, VECO grid; Nissin EM, Tokyo, Japan). The sections on grids were incubated with anti-LC3 antibody (1:50) diluted in 1% bovine serum. The grids were then exposed to a 5-nm colloidal gold anti-rabbit antibody (1:50, Sigma-Aldrich Corp.) for 1 hour. After fixation with 2% glutaraldehyde, the grids were incubated with uranyl acetate and examined by using with a TEM (JEOL).

**Statistical Analysis**

Data are expressed as mean ± SEM. Differences among groups were analyzed using 1-way ANOVA, followed by Dunnett’s post hoc test. A probability value was considered statistically significant when P < 0.05.

**Results**

**Effects of Ripasudil on TNF-Induced Axon Loss**

Compared with degenerative axons in the TNF-treated group (Fig. 1B), ripasudil showed a slight protective tendency in the 2 and 20 pmol-treated groups (Figs. 1C, 1D), although quantitative analysis showed no statistically significant (2 pmol: n = 4, P = 0.4289 versus TNF alone; 20 pmol: n = 5, P = 0.1455 versus TNF alone; Fig. 1F). However, a significant protective effect (74.7% protection) of ripasudil 200 pmol was observed (n = 6, P = 0.0132 versus TNF alone; Figs. 1E, 1F).

**Effects of Ripasudil on p62 and LC3-II Expression**

Ripasudil totally prevented upregulation of p62 induced by TNF (Fig. 2A). In addition, ripasudil alone injection decreased p62 levels (Fig. 2B) and markedly increased LC3-II levels (Fig. 2C) compared with the basal levels. Since we previously found no significant change in LC3-II levels in optic nerves after TNF injection, we compared LC3-II levels after TNF plus ripasudil injection with levels after TNF injection alone. We found a remarkable increase in LC3-II levels after TNF plus ripasudil injection compared with levels after TNF injection alone (Fig. 2D).

In the present study, we also examined these protein levels 2 weeks after intravitreal injection, when axon loss becomes noticeable. Similar to the findings at 1 week, a significant increase in p62 after TNF injection was observed, which was prevented by ripasudil (Fig. 3A). Injection of ripasudil alone also decreased p62 levels (Fig. 3B). Although the effect was weaker than that at 1 week, ripasudil alone also increased LC3-II levels at 2 weeks (Fig. 3C). No change in LC3-II levels was seen following TNF injection, but a significant upregulated LC3-II level was seen after TNF plus ripasudil injection compared with TNF injection alone (Fig. 3D).

**Effects of Ripasudil on Electron Microscopic Findings in Optic Nerves**

In the PBS group, numerous microtubules and neurofilaments were present inside axons, along with some mitochondria (Fig.
We confirmed degenerative changes including delamination of myelin; myelin disorganization (Fig. 4B); degenerating axons; and disorganization of the microtubules (Fig. 4C) in the TNF group. However, the TNF plus ripasudil group showed ameliorated degenerative changes along with preservation of myelin and neurofilament structures (Figs. 4D, 4E). Moreover, we found some autophagosomes after the TNF plus ripasudil injection (Figs. 4D, 4E). When we counted autophagosomes in optic nerve axons, there was a significant increase after TNF plus ripasudil compared with TNF alone (Fig. 4F). Although glial area was more obvious in the TNF group compared with other two groups, the number of autophagosomes in glia did not differ among these groups (Fig. 4G). Morphologically similar autophagosomes apparently contained gold particles (Figs. 4H, 4I), but mitochondria displayed no immunogold labeling of LC3 after ripasudil injection (Fig. 4J).

**Immunohistochemistry of LC3 in Optic Nerves**

As in our previous study, we observed obvious colocalization of LC3 and neurofilaments, indicating that LC3 exists in optic nerve axons (Fig. 5A). Ripasudil injection showed increased immunoreactivity of LC3 at 1 and 2 weeks (Figs. 5B, 5C). We also confirmed LC3-immunoreactivity inside neurofilament-positive fibers (Figs. 5A–C).
Figure 2. Protein expression in optic nerves 1 week following intravitreal injection. Immunoblotting values are normalized to β-actin. (A) p62 expression after injection of PBS, TNF, or TNF + ripasudil. Values are expressed as percentages of control and represent mean ± SEM. n = 5. *P < 0.05. (B) p62 expression after injection of PBS or ripasudil. n = 5. **P < 0.005. (C) LC3-II expression after injection of PBS or ripasudil. n = 4. *P < 0.05. (D) LC3-II expression after injection of TNF or TNF + ripasudil. Values are expressed as percentages of TNF injection values. n = 5. *P < 0.05.
**Figure 3.** Protein expression in optic nerves 2 weeks following intravitreal injection. (A) p62 expression after injection of PBS, TNF, or TNF + ripasudil. Values are expressed as percentages of control and represent mean ± SEM. n = 4. *P < 0.05, **P < 0.005. (B) p62 expression after injection of PBS or ripasudil. n = 5. *P < 0.05. (C) LC3-II expression after injection of PBS or ripasudil. n = 5. *P < 0.05. (D) LC3-II expression after injection of PBS, TNF, or TNF + ripasudil. n = 4. *P < 0.05.
DISCUSSION

The present study found a modest but significant protective effect of ripasudil against axon damage. A previous study demonstrated that systemic ripasudil showed a protective effect on RGCs in optic nerve crush. That study found that optic nerve crush upregulated the retinal Nox1 mRNA level, which was prevented by the systemic administration of ripasudil. Moreover, that study found that ripasudil did not inhibit reactive oxygen species (ROS) production directly but...
suggested that it does so through an indirect mechanism.\textsuperscript{22} ROCK inhibitors were shown to exert beneficial effects in some types of neurons.\textsuperscript{23–25} One hypothesis for that mechanism is that the protective effects are directly due to ROCK inhibition. Following optic nerve crush, substantial increased ROCK expression in the retina was demonstrated in rats\textsuperscript{26} and rabbits\textsuperscript{27}; and Y-39983 and fasudil, a ROCK inhibitor, suppressed those increases and exerted neuroprotection.\textsuperscript{26,27} As indirect ROCK inhibition, a recent study has shown that the novel ROCK inhibitor L-F001 prevented PC12 cell death through attenuating endoplasmic reticulum stress.\textsuperscript{28} Thus, it is possible that the protective effects of ripasudil may also be related to other unknown mechanisms.

When autophagic clearance is perturbed, p62 may accumulate along with increased numbers of autophagic vacuoles in degenerating neurons.\textsuperscript{8} One recent study has reported that p62 transfection resulted in mitochondrial dysfunction and progressive neurodegeneration in the rat brain.\textsuperscript{9} Consistent with our previous results,\textsuperscript{12} the current study also confirmed upregulated p62 levels in optic nerve degeneration, which was prevented by ripasudil. P62 may correlate with autophagic flux, and decreases in its level indicate autophagy induction.

**FIGURE 5.** Immunohistochemistry for LC3 in optic nerves. Apparent colocalization of LC3 with neurofilament was seen in the (A) PBS group. LC3 immunoreactivity increased at (B) 1 week and (C) 2 weeks after ripasudil injection. High-magnification images (inset) show the LC3-immunosignal inside nerve fibers (arrowheads). Scale bar: 50 and 25 μm (inset).
while increases indicate autophagy inhibition. Because the present study found that ripasudil diminished p62 expression compared with the basal levels, it could be hypothesized that ripasudil causes autophagy induction. We also found that ripasudil enhanced LC3-II levels. This is in agreement with the recent findings that fasudil increases LC3-II protein levels in SH-SY5Y cells and increases LC3-II protein levels in primary murine cortical neurons. The latter study also demonstrated that the ROCK inhibitor SR3677 upregulated LC3-II levels and diminished p62 levels in primary murine cortical neurons, suggesting that ROCK inhibition leads to autophagy induction. We observed LC3-immunoreactivity inside neurofilament-positive optic nerve fibers as well as autophagosomes containing immunogold labeling with LC3 inside axons. Taken together with our electron microscopic analysis demonstrating that ripasudil increased autophagosome numbers inside axons after TNF injection, it is plausible that ripasudil exerts axonal protection with autophagy induction. These findings are in line with a previous study indicating that the downregulation of ROCK2 ameliorated crush-induced axonal damage and that the knockdown of ROCK2 diminished p62 levels and upregulated LC3-II levels in primary RGCs in rats.

In summary, this study suggests that ripasudil has an axonal-protective effect in TNF-induced optic nerve damage with enhanced intra-axonal autophagy.

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