Preventive Effects of Rapamycin on Inflammation and Capillary Degeneration in a Rat Model of NMDA-Induced Retinal Injury

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Inhibitors of the mammalian target of rapamycin (mTOR) have been shown to protect against neuronal injury, but the mechanisms underlying this effect are not fully understood. The present study aimed to examine the effects of rapamycin, an inhibitor of the mTOR pathway, on inflammation and capillary degeneration in a rat model of N-methyl-D-aspartate (NMDA)-induced retinal neurotoxicity. Inflammation and capillary degeneration were evaluated by counting the numbers of CD45-positive leukocytes and Iba1-positive microglia, and by measuring the length of empty basement membrane sleeves, respectively. Marked increases in the numbers of leukocytes and microglia were observed 1 d after intravitreal injection of NMDA (200 nmol), and significant capillary degeneration was observed after 7 d. These NMDA-induced changes were significantly reduced by the simultaneous injection of rapamycin (20 nmol) with NMDA. These results suggest that rapamycin has preventive effects on inflammation and capillary degeneration during retinal injury.

Key words endothelial cell; excitotoxicity; leukocyte; mammalian target of rapamycin; microglia

Glutamate is a major excitatory neurotransmitter, and excessive extracellular levels of glutamate cause neuronal cell death. The neurotoxicity induced by elevated glutamate levels is implicated in some ocular diseases, including diabetic retinopathy and glaucoma.1–3 In many cases, glutamate-induced neurotoxicity has been predominantly attributed to overstimulation of N-methyl-D-aspartate (NMDA) receptors.4,5 In addition to direct effects on neurons, indirect effects, such as upregulation of pro-inflammatory cytokines and recruitment of leukocytes into the retina, are involved in NMDA-induced retinal neuronal damage.6,7 Recent studies have shown that rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), exerts neuroprotective and axon growth-promoting effects during neuronal injury.8,9 The mTOR is a serine/threonine kinase that regulates a wide array of cellular functions, including cell proliferation and cell-cycle control.10 There are two distinct functional mTOR complexes, mTOR complex 1 and 2 (mTORC1 and mTORC2). Rapamycin inhibits mTORC1, which prevents phosphorylation of at least 2 well-characterized effectors: the p70S6 kinases (S6K1 and S6K2) and eIF4E-binding proteins (4E-BP1).11 Inactivation of mTORC1 can cause autophagy, which has an important role in cellular homeostasis.12 A role for autophagy has been suggested as a neuroprotective mechanism, whereby it enhances the clearance of harmful protein aggregates.13 In addition, rapamycin could prevent inflammatory responses during brain injury, including activation of microglia.14 Thus, multiple mechanisms might be involved in the neuroprotective effects of rapamycin.

In the present study, we examined the effects of rapamycin on inflammation and capillary degeneration in a rat model of retinal neurotoxicity induced by overstimulation of NMDA receptors.

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MATERIALS AND METHODS

Animals Male Sprague-Dawley rats weighing 220–240 g were maintained on a standard diet (Oriental Yeast Co., Ltd., Tokyo) and tap water ad libitum in a room with a constant temperature (22±2°C) and humidity (55±5%) and a 12-h light/dark cycle. All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research, and the Regulations for the Care and Use of Laboratory Animals in Kitasato University adopted by the Institutional Animal Care and Use Committee of Kitasato University.

Treatments The animals were divided into the two groups: NMDA (n=15) and NMDA+rapamycin (n=15). Rapamycin (LC Laboratories, Woburn, MA, U.S.A.) and NMDA (Nacalai Tesque, Kyoto, Japan) were dissolved in 100% dimethyl sulfoxide (DMSO). Under general anesthesia with 50 mg/kg pentobarbital sodium (Nacalai Tesque), rapamycin (20 nmol) or vehicle (DMSO), mixed with 200 nmol of NMDA in a total volume of 5 μL, was injected into the vitreous cavity of one eye. The same volume of vehicle (5 μL 100% DMSO) was injected into the vitreous cavity of the other eye as a control. The dose of rapamycin was selected based on our previous study showing that 20 nmol/eye was a maximum effective dose.8

Vascular Perfusion One day or 7 d after intravitreal injection of the test compounds, systemic vascular perfusion was performed as previously described.15,16 Briefly, the rats were deeply anesthetized with pentobarbital sodium. The vasculature of each rat was perfused with 1% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 4 min at a pressure of 120 mmHg. The perfusion was performed through the aorta via a cannula in the left ventricle. The right atrium was incised to create an exit route for the fixative.
Immunohistochemical Staining of Retinal Cross-Sections To assess the status of retinal inflammation, we performed immunohistochemical staining of retinal cross-sections for leukocytes and microglia as previously described. Briefly, the eyes were removed after systemic perfusion and were stored in the fixative for 1 h at 4°C. The eyes were rinsed several times with PBS, infiltrated overnight with 30% sucrose in PBS at 4°C, and frozen in an optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, U.S.A.). Tissue sections with a thickness of 16 µm were cut with a cryostat and were dried on glass slides. The sections were rinsed to remove the OCT compound and were subsequently incubated in blocking solution (5% normal hamster serum) in PBS containing 0.3% Triton X-100 (PBS-T) for 0.5 to 1 h at room temperature. The tissue sections were incubated with primary antibodies overnight at room temperature. Leukocytes and microglia were labeled using a mouse monoclonal anti-CD45 antibody (1:100; BD Biosciences, San Jose, CA, U.S.A.) and a rabbit polyclonal anti-Iba1 antibody (1:500; Wako, Pure Chemical Industries, Ltd., Osaka, Japan), respectively. After incubation with the primary antibodies, the sections were rinsed with PBS-T and incubated for 5 h at room temperature with 2 species-specific secondary antibodies (1:400; Jackson ImmunoResearch, West Grove, PA, U.S.A.) that were diluted in PBS-T. The sections were rinsed in PBS-T and were mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, U.S.A.). Sections incubated in the absence of primary antibodies were used as controls; these sections were processed and evaluated for specificity or background levels of staining.

Immunohistochemical Staining of Retinal Whole-Mounts To assess the damage to retinal blood vessels, we performed immunohistochemical staining of retinal whole-mounts for endothelial cells and vascular basement membrane as previously described. After the systemic perfusion was performed as described above, the eyes were removed and stored in fixative for 24 h at 4°C. Each retina was separated from the lens, vitreous, and pigment epithelium using 4 radial cuts and then incubated in blocking solution (5% normal hamster serum) in PBS-T for 0.5 to 1 h at room temperature. The retinas were incubated with primary antibodies overnight at room temperature. Vascular endothelial cells and basement membranes were labeled using mouse monoclonal anti-rat endothelial cell antigen (RECA)-1 antibody (1:200; Serotec, Oxford, U.K.) and rabbit polyclonal anti-type IV collagen antibody (1:8000; Cosmo Bio Co., Tokyo, Japan), respectively. After several rinses with PBS-T, the retinas were incubated for 5 h at room temperature with 2 species-specific secondary antibodies (1:400; Jackson ImmunoResearch) that were diluted in PBS-T. The retinas were then rinsed with PBS-T, and retinal flat-mounts were prepared using a fluorescence mounting medium (Vectashield, Vector Laboratories).

Image Analysis Images were obtained using a fluorescent microscope system BZ-9000 (Keyence, Osaka, Japan) or a confocal laser-scanning microscope LSM 510 Meta (Zeiss, Oberkochen, Germany). To count the CD45- and Iba1-positive cells, images of the mid-peripheral region of the retina were obtained from each retinal section. The numbers of CD45- and Iba1-positive cells present in the GCL and IPL of each retina were counted. Cell counts were normalized to the total counting area (the area of the GCL and the IPL) from each section of retina and the average number was obtained from 3 sections of each retina. To measure the length of the empty basement membrane sleeves, 5 images of the mid-peripheral region of the retina (fields at 10×, corresponding to a retinal area of 1.58 mm² each) were obtained from each retinal flat-mount. All of the morphometric analyses were performed in a blind manner.

Data Analysis Statistical comparisons were performed using an ANOVA, followed by Tukey’s post hoc test (GraphPad, San Diego, CA, U.S.A.). A p value of less than 0.05 was considered statistically significant. All values are presented as the mean±S.E.M.

RESULTS

Figure 1 shows representative photomicrographs of retinal cross-sections labeled with antibodies for leukocytes (CD45) and microglia (Iba1). In vehicle-treated control retina, few CD45-positive cells were observed (Fig. 1Aa), whereas Iba1-positive microglia exhibited ramified morphology (Fig. 1Aa). However, 24 h after intravitreal injection of NMDA (200 nmol/eye), both CD45- and Iba1-positive cells increased abundantly and Iba1-positive cells exhibited a transformation from the ramified form to an amoeboid shape (Figs. 1Ab, Ab'). Some of the CD45-positive cells were co-stained with Iba1 in NMDA-treated retina (Fig. 1Ab'). Simultaneous injection of rapamycin with NMDA significantly decreased the number of CD45-positive cells (Figs. 1Ac, B) and Iba1-positive cells (Figs. 1Ac', C). We did not notice any differences in CD45- or Iba1-positive cells between naïve and vehicle-treated retinas (Supplementary Figure 1). These results indicate that rapamycin prevents the leukocyte infiltration and microglial activation observed in NMDA-treated retina.

Figure 2 shows representative photomicrographs of retinal flat-mounts labeled with antibodies for endothelial cells (RECA) and vascular basement membranes (type IV collagen). The immunoreactivity of type IV collagen was almost completely colocalized with RECA staining of blood vessels in control retina (Figs. 2Aa, Aa', Aa''), but the number of empty vascular basement membrane sleeves increased 7 d after NMDA treatment (Figs. 2Ab, Ab', Ab''). The total length of empty basement membrane sleeves was significantly increased after NMDA injection, and the increase was abolished by rapamycin (Figs. 2Ac', B).

DISCUSSION

The present study demonstrates that rapamycin has preventive effects on inflammatory responses and vascular injury in a rat model of NMDA-induced retinal neurotoxicity. Our previous study has shown that rapamycin exerts protective effects on retinal neuronal cells in the same model. Thus, rapamycin exerts multiple beneficial effects during retinal injury.

Previous reports have shown immunoreactivity for pS6, a downstream indicator of mTORC1 activity, in retinal ganglion cells (RGCs) in the ganglion cell layer (GCL) and other retinal cells present in the inner plexiform layer (IPL) and inner nuclear layer (INL), such as Müller cells and microglia. The pS6 immunoreactivity was markedly increased at relatively early time points (2–6 h) after NMDA injection and was totally prevented by co-injection of rapamycin. At present, the role
of the increased mTOR activity remains unclear. However, the findings suggest that rapamycin acts on multiple retinal cell types that activate the mTOR pathway during NMDA-induced retinal injury.

Infiltration of leukocytes and activation of microglia are commonly observed in the injured retina. Infiltrated leukocytes release oxidants and proteases, which induce inflammatory reactions that further damage the injured retina. In NMDA-treated retina, leukocyte adherence to the vascular endothelium and subsequent infiltration into retinal tissues are proposed as indirect mechanisms that contribute to retinal damage. On the other hand, the exact role of microglial activation in NMDA-treated retina remains unclear, but microglia have been shown to play dual roles in the progression of neurodegenerative disorders. Thus, suppression of inflammatory responses, such as infiltration of leukocytes and activation of microglia, might prevent secondary retinal damage. In the present study, we found increases in CD45-positive cells and Iba1-positive cells, and the morphologic transformation of Iba1-positive cells from the ramified form to an amoeboid shape 24 h after NMDA injection. In addition, the finding that some of the Iba1-positive cells were co-stained with CD45 in NMDA-treated retina suggests that the increase in Iba1-positive cells may be partly due to the differentiation of recruited CD45-positive cells to microglia. These responses were attenuated by co-injection of rapamycin with NMDA. The reduction of inflammatory responses in the injured retina by rapamycin may contribute to the prevention of additional retinal damage.

Retinal vascular damage is also a common observation in the injured retina. Despite the incomplete attenuation of RGC loss or inflammation, rapamycin almost completely prevented retinal vascular damage, suggesting that it may exert a protective effect against vascular damage. However, an alternative explanation is possible; that is, decreasing neuronal cell death and inflammation with rapamycin may be sufficient to preclude the level of retinal neural degeneration necessary to induce retinal vascular degeneration. Indeed, we found that significant neuronal cell loss was observed without an increase in capillary degeneration 2 d after NMDA treatment. There may be a certain threshold level of retinal neural degeneration, in terms of the degree and duration, before the onset of retinal vascular degeneration.

In conclusion, we found that rapamycin affords protection from inflammation and vascular injury in NMDA-treated retina. Rapamycin can be used as an immunosuppressant and mitigate leukocyte infiltration and macrophage/microglia accumulation associated with RGC death. Thus, part of the neuroprotective effects of rapamycin may be due to its anti-inflammatory effects. The precise mechanisms underlying the
beneficial effects remain unclear; however, our results indicate that rapamycin may be considered as a candidate for neuroprotective interventions in retinal diseases associated with glutamate-induced excitotoxicity.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES