P7C3 Suppresses Neuroinflammation and Protects Retinal Ganglion Cells of Rats from Optic Nerve Crush

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PURPOSE. To determine whether P7C3-A20 can inhibit the phosphorylation of the mammalian target of rapamycin (mTOR), depress neuroinflammation, and protect retinal ganglion cells (RGCs) of rats from optic nerve crush (ONC).

METHODS. The left optic nerve was crushed, and 5.0 mg/kg/d of P7C3-A20, 1.0 mg/kg/d of rapamycin, or their vehicle was injected intraperitoneally for 3 consecutive days beginning 1 day before the ONC. The protective effects on the RGCs were determined by immunohistochemical staining for Tuji-1. The level of phosphorylated mTOR was determined by immunoblotting. The neuroinflammation in the optic nerve was determined by changes in the expression of CD68, TNF-α, MCP-1, and iNOS.

RESULTS. The density of Tuji-1-stained cells in the control was 2010 ± 81.5/mm² and 1842 ± 80.4/mm² on days 7 and 14 after the sham operation. These levels were lower at 995 ± 122/mm² and 450 ± 52.4/mm² on days 7 and 14 after the ONC, respectively. Rapamycin and P7C3-A20 preserved the density at significantly higher levels on both days (P < 0.05, Scheffe test). The level of phosphorylated mTOR increased by 1.56-fold above the control level on day 7. Rapamycin and P7C3 significantly lowered the level of phosphorylated mTOR to 0.89-fold and 0.67-fold of the control, respectively. There was an accumulation of CD68-positive cells that were immunoreactive to TNF-α at the crush site. The expression of MCP-1 and iNOS was increased chiefly in the astrocytes around the lesion. These inflammatory events were suppressed by both rapamycin and P7C3.

CONCLUSIONS. P7C3-A20 can inhibit mTOR phosphorylation in the crushed optic nerve, which may suppress neuroinflammation and preserve the RGCs.

Keywords: P7C3, rapamycin, mTOR, optic nerve crush, NAD

P7C3, a neuroprotective compound, was discovered by comprehensive in vivo screening during neurogenesis.1 This compound activates nicotinamide phosphoribosyltransferase (NAMPT),2 a self-limiting enzyme, which is involved in the nicotinamide adenine dinucleotide (NAD) salvage pathway. NAD mediates the protective activities of the NAMPT gene that delays axonal degeneration from various insults including high intraocular pressures (i.e., glaucoma).3 We have shown that intraperitoneal injections of P7C3-A20, an active form of P7C3, rescued retinal ganglion cells (RGCs) from retrograde degeneration after optic nerve crush (ONC) in rats.4

Astrocytes, the main glial cells in the central nervous system (CNS), become reactive when the optic nerve is injured. This step is important in the repair process5 because the reactive astrocytes express several cell adhesion molecules and chemokines.6,7 In addition, a recruitment of microglia and macrophages occurs at the site of the lesion that induces the formation of proinflammatory molecules including TNF-α and nitric oxide.8 These changes lead to secondary axonal damage.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates protein translation in response to changes of the cellular environment, for example, nutrition, oxygen concentration, and other stress factors. mTOR consists of two different protein complexes, mTORC1 and mTORC2, and rapamycin selectively inhibits only mTORC1.9 mTOR is closely associated with the innate immune system,10 and it is a key regulator of glial activity, cellular proliferation, and cytokine release.11 Thus, inhibition of the mTOR activity may reduce neuroinflammation.11 On the other hand, it has been reported that activation of the mTOR signaling pathways may have neuroprotective action in RGCs.12

NAD is a coenzyme found in all living cells and is involved in many biological processes including the production of ATP as an electron carrier by changing its redox state from NAD to NADH. When the cellular energy level is low, for example, starvation and exercise, there is an increase in the ratio of AMP/ATP, which activates 5′ AMP-activated protein kinase (AMPK). This then causes an increase in the NAD level and consequently an increase in the NAD/NADH ratio.13 Thus, the intracellular NAD/NADH ratio can interact with the nutrient and redox states of the cells. On the other hand, AMPK inhibits the activity of mTOR kinase,14 which suggests that there may be a link between the NAD and mTOR signaling pathways.

We hypothesized that P7C3 will depress neuroinflammation through the inhibition of mTOR activity and thus protect RGCs from degeneration after ONC. To test this hypothesis, we crushed the optic nerves of rats, and we injected either rapamycin (1 mg/kg), P7C3-A20 (5 mg/kg), or their vehicle (pure dimethyl sulfoxide [DMSO], 100 µL) intraperitoneally for 3 days beginning 1 day before the ONC. Their effects were...
determined by counting the number of Tuj-1–stained cells on days 7 and 14 after the ONC. The levels of phosphorylated mTOR in the optic nerve and the retina were determined by immunoblot. The degree of neuroinflammation in the optic nerve was determined by the levels of expression of CD68, MCP-1, TNF-α, and iNOS. In addition, the levels of phosphorylated Akt and CHOP, an endoplasmic reticulum (ER) stress marker,15,16 were determined by immunoblot analyses to examine the neuroprotective action of P7C3 on the RGCs.

**METHODS**

**Animals**

Nine-week-old, male Wistar rats were purchased from Japan SLC (Shizuoka, Japan) and housed in an air-conditioned room with a temperature of approximately 23°C and humidity of 60%. The room lights were on a 12:12 light to dark cycle. All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental protocol was approved by the Committee of Animal Use and Care of the Osaka Medical College (No. 28024). A total of 64 rats were used.

**Chemicals**

Unless noted, all chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). P7C3-A20, an active variant of P7C3,2 was purchased from AdooQ Bioscience (Irvine, CA, USA). P7C3 at ≥5 mg/kg has proneurogenic activity in rats1 and we chose this concentration for these experiments. Rapamycin was purchased from Tokyo Chemical Industry (Tokyo, Japan), and it has been shown that 1 mg/kg of rapamycin is neuroprotective against traumatic brain injury.17 Thus, we used 1 mg/kg of rapamycin for this study. Both P7C31 and rapamycin17 can cross the blood–brain barrier.

**Anesthesia and Euthanasia**

All surgeries were performed under general anesthesia induced by an intraperitoneal injection of a mixture of medetomidine (0.75 mg), midazolam hydrochloride (4.0 mg), and butorphanol tartrate (5.0 mg/kg body weight). Rats were euthanized in a 1.0 mg/kg/d of rapamycin or its vehicle, 100 µL of saline.

**Optic Nerve Crush**

Animals were anesthetized as described, and an incision was made along the midline of the skull to expose the superior surface of the left eye. The superior rectus muscle was incised to expose the left optic nerve, and the left optic nerve was crushed 2 mm behind the eye with forceps for 10 seconds.18 Care was taken not to occlude the blood vessels and cause retinal ischemia. We confirmed that the retinal circulation was not blocked by indirect ophthalmoscopy and also verified this method described in detail by Winkler.20 Then the skull and cerebral hemispheres were removed to reveal the optic nerves, and the optic nerves were carefully isolated including the crushed site.

**Isolation of Retinas and Optic Nerves for Immunohistochemistry**

Rats were deeply anaesthetized as described and perfused through the heart with saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer of pH 7.4. The retinas were carefully removed from the eyes according to the methods described in detail by Winkler.20 Then the skull and cerebral hemispheres were removed to reveal the optic nerves, and the optic nerves were carefully isolated including the crushed site.

**Labeling Retinal Ganglion Cells**

Earlier studies have shown that axotomy causes a reduction in the number of RGCs in a delayed fashion; the number of RGCs remains unchanged for 5 days after the injury and then abruptly decreases to 50% on day 7 and to less than 10% on day 14.21 Thus for our study, the number of surviving RGCs was determined on days 7 and 14 after the ONC.

Retinas were isolated after transcardiac fixation as described, and they were sandwiched between nylon mesh sheets (N0255HD; NBC Meshtec, Tokyo, Japan) and postfixed in 4% PFA in PBS overnight. After washing in PBS and blocking in PBS containing 1.0% BS and 0.3% Triton X-100, the retinas were incubated with Alexa 488–conjugated mouse monoclonal neuron-specific class III β-tubulin (Tuj-1, 1:500) antibody (Covance, Princeton, NJ, USA). Tuj-1 is a marker for RGCs.22,23 The retinas were placed in the same medium overnight at 4°C and were washed with PBS and coverslipped the next morning.

To determine the number of RGCs that were Tuj-1 positive, the stained flat mounts were photographed with a fluorescent microscope (BZ X700; Keyence, Osaka, Japan). Eight areas (0.48 × 0.48 mm) from the four quadrants of the retina at 1.0 and 1.5 mm from the margin of the optic disc were photographed. All of the Tuj-1–positive cells in an area of 0.2 × 0.2 mm at the center of each image were counted by using the ImageJ program (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

The mean density of the Tuj-1–positive cells per square millimeter was calculated, and the loss of RGCs was estimated by comparing the density in the retinas of animals receiving either rapamycin, P7C3-A20, or their vehicle after the ONC to that of retinas from sham controls. The number of Tuj-1–positive cells was counted by one observer (SM) who was masked as to whether it was from an experimental or a sham animal.

**Immunohistochemistry of Optic Nerve**

The optic nerves were isolated for immunohistochemistry on day 7 after the ONC as described. The isolated optic nerves were postfixed in 4% PFA in PBS overnight. After washing with PBS, the tissues were immersed in 30% sucrose for 2 days at 4°C and then embedded in OCT compound (Sakura Finetek, Torrance, CA, USA). Then, 5-µm frozen sections were cut with a cryostat. After blocking the sections in 1.0% normal goat or donkey serum plus 1.0% BSA and 0.1% Triton-X 100 in PBS, the sections of the optic nerves were incubated with the primary antibodies of mouse anti-CD68 antibody (1:500; Serotec, Oxford, UK), goat polyclonal anti-MCP-1 antibody (1:200; Santa Cruz, Dallas, TX, USA), mouse monoclonal or rabbit polyclonal anti-GFAP antibody (1:500; Merck Millipore, Billerica, MA, USA), or rabbit polyclonal anti-iNOS antibody overnight at 4°C. The sections were then incubated overnight at 4°C in Alexa 594 or Alexa 488 conjugated to the appropriate...
secondary antibodies (Invitrogen, Carlsbad, CA, USA) diluted by 1:500.

The sections were photographed with a fluorescence microscope (BX-700) or a confocal laser microscope (TCS SP8; Leica, Wetzlar, Germany).

Changes in Expression of CD68, MCP-1, and TNF-α Genes in Optic Nerve

We previously showed that ONC caused an accumulation of CD68+ cells, microglia and macrophages, at the site of the lesion. To quantify the degree of inflammation after the injury in each experimental group, we determined the changes in the expression of CD68 and MCP-1 in the optic nerve by real-time PCR (RT-PCR) on day 7 after the ONC. In addition, the mRNA level of TNF-α was determined because activated microglia and macrophages release inflammatory cytokines.

For these analyses, the optic nerves (approximately 4 mm including the crushed site at the center) were excised after euthanasia, and they were homogenized in lysis buffer. Total RNA was extracted by using a NucleoSpin RNA kit (TaKaRa, Ohtsu, Shiga, Japan), and the RNA quality and quantity was assessed with a BioSpectrometer (Eppendorf, Hamburg, Germany).

Purified RNA was reverse transcribed with the PrimeScript RT reagent and gDNA Eraser kits according to the manufacturer’s instructions (TaKaRa). Quantitative real-time PCR analysis was performed with the Thermal Cycler Dice Real Time system TP870 (TaKaRa), Premix Ex Taq (Probe qPCR; TaKaRa), and the TaqMan Gene Expression Assays for targeted genes (Applied Biosystems, Foster City, CA). The rat TaqMan Gene Assays for CD68 (Rn01495634_g1), MCP-1 (Rn00580555_m1), and TNF-α (Rn0152859_g1) were used. Amplicons were detected with the relevant probes tagged with an MGB quencher and an FAM dye. All reactions were run with the following cycling parameters: 30 seconds at 95°C followed by 40 cycles at 95°C for 5 seconds, and 60°C for 30 seconds. A standard curve of the cycle thresholds was established by using serial dilutions of the cDNA samples. The relative quantities of mRNAs were calculated with $\Delta \Delta C$ (Hs99999901_s1) serving as an internal control.

Immunoblotting

Immunoblot analyses were used to determine the effects of rapamycin and P7C3 on the expression of mTOR in the crushed optic nerve. On activation, mTOR is phosphorylated at several sites including at Ser2448. Thus, the levels of phosphorylated mTOR (Ser2448) in the optic nerve were determined. In addition, the levels of phosphorylated mTOR (Ser2448) and the supernatant was used to determine the total protein concentration by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Samples were separated on a 7.5% SDS-polyacrylamide gel and transblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in TBS-T (pH 7.4, 0.1% Tween 20) followed by overnight incubation with a rabbit polyclonal anti–phosphorylated mTOR (Ser2448; Cell Signaling, Danvers, MA, USA) and mouse monoclonal anti-mTOR (Cell Signaling) at 4°C. In addition, the membrane was incubated with rabbit polyclonal anti-NOS2 (Santa Cruz, Dallas, TX, USA) in the same way. Then, α-tubulin (Merck Millipore, Darmstadt, Germany) was used as an internal control for NOS2, that is, INO8.

The protein bands were made visible by horseradish peroxidase-conjugated appropriate secondary antibodies (Promega, Madison, WI, USA). The signals were intensified with an enhanced chemiluminescence (ECL) plus Western blotting detection system (GE Healthcare, Buckinghamshire, UK). The densities of the bands of proteins were quantified with a luminescent image analyzer (LAS-3000; Fujifilm, Tokyo, Japan). The levels of expression of these proteins were quantified with the embedded software (Multi Gauge version 3.0) and standardized to the control level. The assays were run in triplicate, using the pooled samples.

In addition to the optic nerves, retinas were isolated by Winkler method after euthanasia, and they were subjected to immunoblot to determine the effects of rapamycin and P7C3 on the expression of mTOR and Akt as well as their phosphorylated forms. Furthermore, because ER stress is involved in the mechanisms of death of RGCs after ONC and glaucoma, we determined the expression of CHOP in the retina to assess whether the ER stress is relieved through a reduction of mTOR-driven protein synthesis.

Immunoblot for the phosphorylated mTOR (Ser2448) was performed in the same way as we did for the optic nerve samples. To determine the expression of phosphorylated Akt (Thr308) and CHOP, extracts from retinal samples were separated on a 10% and 14% SDS-polyacrylamide gel, respectively. Rabbit polyclonal anti–phosphorylated Akt (Thr308; Cell Signaling), mouse monoclonal anti-Akt (Santa Cruz), and rabbit monoclonal anti-CHOP (Abcam plc, Cambridge, UK) antibodies were used as primary antibodies for these assays.

RESULTS

Decrease of RGCs After Optic Nerve Crush

Representative photomicrographs of flat-mounted retinas taken approximately 1.0 mm from the optic disc margin on days 7 and 14 are shown in Figures 1a and 1b, respectively. Tuj-1-stained cells were densely arranged in the control (sham), while the number of Tuj-1–positive cells was clearly reduced after the ONC (crush-placebo) on day 7 (Fig. 1a). There appeared to be a larger number of stained cells in animals that received intraperitoneal injections of rapamycin (1.0 mg/kg/d × 3 days; crush-rapamycin) than in the crush-placebo group (Fig. 1a).

The mean ± SD of the number of Tuj-1–stained cells was 2010 ± 81.5/mm² on day 7 in the sham-operated rats (sham control, n = 6), and the mean number was decreased significantly to 995 ± 122/mm² (n = 8) on day 7 in the crush-placebo group (P < 0.01, Scheffe test; Fig. 1c, left). The reduction was significantly depressed to 1432 ± 120/mm² (n = 6) in the crush-rapamycin group (P < 0.01, Scheffe; Fig. 1c, left) and 1593 ± 149/mm² (n = 6) in the crush-P7C3 group (P < 0.01, Scheffe; Fig. 1c, left).

On day 14, there was a further decrease in the number of Tuj-1–stained cells in the crush-placebo retina (Fig. 1b). There was a similar decline from day 7 in the number of stained cells in animals that received rapamycin (crush-rapamycin) or P7C3-A20 (5.0 mg/kg/d × 3 days; crush-P7C3) than in the crush-placebo group (Fig. 1a).

The mean ± SD of the number of Tuj-1–stained cells was 146/mm² on day 7 in the sham-operated rats (sham control, n = 6), and the mean number was decreased significantly to 450 ± 52.4/mm² in the crush-placebo group (n = 4), while it was maintained at 644 ± 146/mm² in the crush-
rapamycin group \((n=4)\) and 625 ± 66.1/mm² in the crush-P7C3 group \((n=4)\). The differences between the crush-placebo and crush-rapamycin groups as well as between the crush-placebo and crush-P7C3 groups were significant \((P=0.03\) and \(0.04,\) respectively; Scheffe). Thus, rapamycin and P7C3-A20 still had protective effects on day 14 after the ONC (Fig. 1c, right).

Immunohistochemistry for the Optic Nerve

The neuroinflammatory events include proliferation and activation of microglia and macrophages, upregulation of cytokine production, and nitrosative stress.8 We performed immunohistologic analyses for proteins associated with these events to determine the degree of neuroinflammation in the

**FIGURE 1.** Representative photomicrographs of flat-mounted retinas stained with Alexa 488-conjugated Tuj-1 antibody demonstrating the retinal damages 7 and 14 days after ONC. (a, b) Tuj-1 labeling on days 7 and 14 after ONC, respectively. Photographs were taken 1.0 mm from the optic disc margin. Scale bar: 100 μm. (c) Plots of the mean density of Tuj-1-positive cells per square millimeter on days 7 (left) and 14 (right). There is a significant reduction in the number of Tuj-1-positive cells after ONC on day 7, which is further decreased on day 14 (crush-placebo). Rapamycin (crush-rapamycin) or P7C3-A20 (crush-P7C3) depressed the reduction on day 7 (left), and these compounds still preserve the density on day 14 (right). Data are the means ± standard deviations (SDs) \(*^*P<0.01, ^*P<0.05;\) Scheffe.)
optic nerve after the ONC. Representative photomicrographs of optic nerve sections after immunohistochemical staining for CD68 and GFAP on day 7 are shown in Figure 2. In each image, the left side is proximal to the eye and the right side is distal to the eye. At the crushed site in the crush-placebo group, the immunoreactivity to GFAP was reduced, and an accumulation of CD68$^+$ cells, microglia and macrophages, was present (Fig. 2b). The number of CD68$^+$ cells at the crush site was clearly lower in the crush-rapamycin (Fig. 2c) and in the crush-P7C3 (Fig. 2d).

Double labeling for CD68 and other inflammatory mediators is shown in Figure 3. CD68 and TNF-$\alpha$ had a high incidence of colocalization (Fig. 3a). This supports the idea that these CD68$^+$ cells, microglia and macrophages, have an ability to secrete TNF-$\alpha$. However, CD68, MCP-1, and iNOS were not highly colocalized (Figs. 3a, 3b).

Next, we sought to determine the cellular sources of MCP-1 and iNOS expression. Double labeling for MCP-1 and GFAP is shown in Figure 4. MCP-1 was expressed more highly at the crush site in the crush-placebo group (Fig. 4a), while the expression seemed to be depressed in the crush-rapamycin group (Fig. 4b) and in the crush-P7C3 group (Fig. 4c). Confocal images of double labeling for MCP-1 and GFAP at the crush site obtained from the crush-placebo group are shown in Figure 4d. While MCP-1 was expressed more strongly at the crush site where expression of GFAP is decreased, these two proteins were highly colocalized, indicating that MCP-1 is chiefly expressed in the astrocytes at the crush site.

Photomicrographs showing the results of double labeling for iNOS and GFAP are shown in Figure 5. The results showed that iNOS was less expressed at the crush site for all experimental groups, and the expression pattern was similar to that of GFAP. However, the iNOS expression appeared to be more enhanced in the astrocytes in the area just distal to the crush site where Wallerian degeneration would proceed in the crush-placebo group (Fig. 5a). Confocal images of double
labeling for iNOS and GFAP in the optic nerve obtained from the crush-placebo group are shown in Figure 5d. The expression of these two proteins was highly colocalized. These findings suggest that MCP-1 was mainly expressed in the astrocytes at the crushed site, while iNOS expression increased in the astrocytes located in the area just distal to the lesion.

Alterations in Expression of CD68, TNF-α, and MCP-1 by Real-Time PCR

We determined the effects of rapamycin and P7C3 on the expressions of the genes of CD68, TNF-α, and MCP-1 quantitatively by RT-PCR. The mRNA levels of these genes in the optic nerves on day 7 after the ONC are demonstrated in Figure 6. The levels are presented relative to that of the sham controls.

The level of the mRNA of CD68 was increased by $1090 \pm 519.8$-fold of the control level after the ONC (crush-placebo). This increase was suppressed by both rapamycin ($P = 0.002$) and P7C3 ($P = 0.004$, Scheffe), and the levels were $60.9 \pm 36.9$- and $123.5 \pm 116.3$-fold, respectively (Fig. 6a).

Because TNF-α is a neurodestructive inflammatory cytokine, its level was also determined. There was a $22.1$-fold increase in the mRNA of TNF-α after the ONC (crush-placebo). This increase was significantly suppressed by either rapamycin ($P < 0.001$) or P7C3 ($P < 0.001$, Scheffe).

Chemokines promote the recruitment of leukocytes. Optic nerve crush caused a 14.4-fold increase in the expression of the mRNA of MCP-1 on day 7 in the crush-placebo group. Both rapamycin and P7C3 suppressed the increase significantly ($P < 0.05$, Tukey), to 5.6-fold by rapamycin and to 5.9-fold by P7C3 (Fig. 6c).

Changes in Expression of mTOR and iNOS in the Optic Nerve

Initially, we determined the temporal changes in the levels of phosphorylated mTOR in the optic nerve by Western blot analyses. The level of phosphorylated mTOR was increased by 1.13-fold on day 1; 0.98-fold on day 2; 1.44-fold on day 4; and 1.59-fold on day 7 relative to the baseline level ($n = 3$ at each time point). The phosphorylated mTOR was higher than the baseline level on days 4 and 7 ($P < 0.01$, Scheffe).

We thus determined the effects of rapamycin and P7C3 on the expression of the phosphorylated mTOR on day 7. There was a 1.56-fold increase of phosphorylated mTOR expression from the sham control on day 7 in the crush-placebo group ($P < 0.001$ versus control, Scheffe). Rapamycin depressed the level to 0.89-fold and P7C3 lowered the level to 0.67-fold of the control level (both $P < 0.001$ versus crush-placebo group, Scheffe; Fig. 7a).

Similar changes were observed in the expression of iNOS (Fig. 7b). There was a 1.93-fold increase of iNOS expression in the crush-placebo group relative to that in the control level ($P = 0.001$, Scheffe). Rapamycin and P7C3 depressed
the increase to 1.54-fold and 1.38-fold from the control level, respectively. These depressive effects in the crush-placebo group were significant ($P = 0.03$ and $P = 0.01$, respectively; Scheffe).

Changes in Expression of mTOR, Akt, and CHOP in Retina

We further determined the effects of rapamycin and P7C3 on the expression of phosphorylated mTOR in the retina by

![Immunohistochemistry for GFAP and MCP-1 in the optic nerve in the crush-placebo (a), crush-rapamycin (b), and crush-P7C3 (c) groups.](https://tvst.arvojournals.org/)

(a) Immunoreactivity to GFAP is weak at the crush site (arrow) where immunoreactivity to MCP-1 is increased as was seen in Figure 2. (b, c) Immunoreactivity to MCP-1 is weak in the animals treated with rapamycin (b) or P7C3 (c). Crush site is indicated by arrow in each photomicrograph. Scale bar: 100 μm. (d) Confocal images at the crush site of crush-placebo group (area indicated by arrowhead in [a]). Immunoreactivity to GFAP and MCP-1 is well colocalized, suggesting that astrocytes at the crush site express MCP-1.
Western blot analyses on day 7. Because there are feedback loops between the mTOR and Akt signaling pathways, the level of phosphorylated Akt (Thr308) was also determined. The data are shown in Figure 8. Consistent with the results obtained from the optic nerve, the level of phosphorylated mTOR (Ser2448) was increased by 1.3-fold in the crush-placebo group ($P < 0.05$ versus control, Scheffe). Rapamycin depressed the level to 0.56-fold and P7C3 lowered the level to

**Figure 5.** Immunohistochemistry for GFAP and iNOS in the optic nerve from crush-placebo (a), crush-rapamycin (b), and crush-P7C3 (c) groups. (a) Immunoreactivity to GFAP is weak at the crush site (arrow), while immunoreactivity to iNOS is intensified in the area just distal to the crush site (arrowhead). (b, c) Immunoreactivity to GFAP is weak at the crush site (arrow) and that to iNOS appears not to be intensified around the crush site in the animals treated with rapamycin (b) or P7C3 (c). Scale bar: 100 μm. (d) Confocal images at the crush site of crush-placebo group (area indicated by arrowhead in (a)). Immunoreactivity to GFAP and iNOS is well colocalized especially around the upper rim area in the image, suggesting that astrocytes express iNOS around the crush site.
0.52-fold of the control level, and these levels were significantly lower (both \( P < 0.001 \), Scheffe) than that from the crush-placebo group (Fig. 7a).

On the other hand, the levels of phosphorylated Akt (Thr308) were decreased by 0.87-fold of the control level after the ONC (crush-placebo, Fig. 8b). Rapamycin increased the level to 1.16-fold and P7C3-A20 elevated the level to 1.23-fold of the control level, and these levels were significantly higher (both \( P < 0.05 \), Tukey) than that from the crush-placebo group (Fig. 8b). However, ONC caused 1.4-fold increase of CHOP expression in the retina over the control level (crush-placebo, Fig. 8c). Rapamycin and P7C3-A20 significantly suppressed the increase (\( P < 0.05 \), Tukey; Fig. 8c).

**DISCUSSION**

Our results showed that ONC enhanced the phosphorylation of mTOR in the optic nerve, and an intraperitoneal injection of rapamycin or P7C3-A20 depressed the phosphorylation. In addition, both rapamycin and P7C3-A20 slowed the loss of RGCs from retrograde degeneration. At the crushed site, there was an accumulation of CD68+ cells that can secrete TNF-\( \alpha \).
The expression of MCP-1 and iNOS was increased chiefly in the astrocytes around the lesion. These inflammatory events were suppressed by both rapamycin and P7C3. In the retina, rapamycin and P7C3-A20 also depressed the phosphorylation of mTOR after ONC, but these compounds increased the levels of phosphorylated Akt. In addition, CHOP was increased in the retina in the crush-placebo group, and the increase was suppressed by both rapamycin and P7C3.

Our results showed that both rapamycin and P7C3-A20 preserved the number of Tuj-1–positive cells on days 7 and 14. A study with retrograde labeling shows that axotomy decreases the RGCs to less than 10% on day 14, while 25% of Tuj-1–positive cells still existed on day 14 in our study. Thus, Tuj-1–positive cells do not necessarily indicate that they are alive. In addition, Tuj-1 is not a specific marker for RGCs. Despite taking these properties into consideration, it can be concluded that these chemicals delay the loss of RGCs from degeneration after ONC for a considerable period of time.

It has been reported that the activity of mTOR is suppressed in the RGCs after optic nerve injury, and the selective activation of mTOR in the RGCs enhances their survival and promotes axonal extension after optic nerve injury. These findings appear to contradict our results but these studies examine the local activation of mTOR in the RGCs by the deletion of a negative regulator, PTEN, or an inhibitor, RTP801, that are delivered through the intravitreal route. However, we did not influence mTOR in the RGCs selectively, and the methodologic differences may account for the discrepancy.

The mTOR signaling pathway plays important roles in the immune system by regulating T-cell proliferation, macrophage polarization, cytokine release, and chemokine expression. Despite these properties into consideration, it can be concluded that these chemicals delay the loss of RGCs from degeneration after ONC for a considerable period of time.

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expression of iNOS. These findings indicate that inhibition of mTOR can depress neuroinflammation in the CNS.

It is well known that neuroinflammation can cause secondary damage to the CNS, where accumulated microglia and macrophages play crucial roles. Axonal injury in the CNS enhances the expression of MCP-1 in the astrocytes and microglia, which then leads to an infiltration of macrophages and leukocytes into the lesion. Although recruited macrophages may play beneficial roles, deletion of MCP-1 can suppress the accumulation of macrophages and decrease the secondary neuronal damage in traumatic brain injury. Our results showed that both rapamycin and P7C3 depressed neuroinflammation by lowering the level of phosphorylated mTOR. Consistently, it has been shown that inhibition of mTOR signaling can attenuate neuronal damage in traumatic and ischemic brain damage, and spinal cord injuries. The increased expression of TNF-α and iNOS that was found in this study probably enhanced the mitochondrial damage and caused oxidative and nitrosative stress, which may lead to neuroinflammation and secondary axonal damage. Because rapamycin selectively inhibits mTORC1, it is reasonable to consider that the anti-inflammatory effects of P7C3 are mediated by inhibition of mTORC1, although an involvement of mTORC2 cannot be completely eliminated.

Several mechanisms have been identified for NAD to prevent axonal degeneration such as the maintenance of mitochondrial function, ionic homeostasis, and suppression of oxidative stress. On the other hand, NAD can regulate the activities of mTOR1, and Sirt1 can inhibit the mTOR signaling pathway. Because P7C3 is known to activate the NAMPT gene, the P7C3-induced increase of NAD may inhibit the mTOR signaling pathway. We have reported that P7C3-A2O preserves the NAD level and increases the mRNA level of Sirt1 in the crushed optic nerve. In addition, Kitaoka et al. have suggested that overexpression of NMNAT3 has neuroprotective effects against axonal damage in the optic nerve through an improvement of autophagic flux by modulating the level of mTOR.

In addition to the inhibitive effects on neuroinflammation in the optic nerve, these compounds may have independent beneficial effects on the RGCs. We found that the phosphorylated Akt levels in the retina were higher in the crush-rapamycin and crush-P7C3 groups than in the crush-placebo group, while the phosphorylated mTOR levels were oppositely higher in the crush-placebo group than in the crush-rapamycin and crush-P7C3 groups. This interaction may be due to a relief of negative feedback loop from the mTOR signaling pathway. Akt is closely associated with cellular survival, and the levels of phosphorylated Akt are decreased in the eyes with experimental glaucoma and after excitotoxicity in RGCs. Thus, enhanced phosphorylation of Akt in the retina may have beneficial effects on the RGCs. We also found that the expression of CHOP was increased in the retina in the crush-placebo group, indicating that ER stress is involved in the mechanisms of loss of RGCs after ONC. Attenuation of protein synthesis through the inhibition of mTOR phosphorylation may decrease the amount of misfolded proteins, which may reduce the ER stress in the retina. These mTOR-related events may also contribute to the delay of RGCs’ decrease in our study.

There were limitations in this study. One limitation was that we did not determine how the mTOR functions changed in the RGCs after the optic nerve crush and how the P7C3-A2O affected the functions. In addition, we did not determine the mechanisms of how P7C3 inhibited mTOR function. These issues need to be determined in future studies.

In conclusion, the neuroprotection of RGCs by P7C3 probably involves inhibition of the mTOR phosphorylation and depression of neuroinflammation. In addition, P7C3 may affect Akt phosphorylation and decrease ER stress through a mechanism related to inhibition of mTOR phosphorylation. Thus, P7C3 appears to have other effects than have been reported.

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References


