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Axonal protection by modulation of p62 expression in TNF-induced optic nerve degeneration

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HIGHLIGHTS

- p62 was upregulated in the optic nerve after intravitreal injection of TNF.
- Treatment with p62 siRNA exerted a partial but significant protective effect.
- Rapamycin both decreased p62 levels and exerted substantial axonal protection.

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ABSTRACT

p62, which is also called sequestosome 1 (SQSTM1), plays a critical role in neuronal cell death. However, the role of p62 in axonal degeneration remains unclear. We evaluated whether the modulation of p62 expression may affect axonal loss in tumor necrosis factor (TNF)-induced optic nerve degeneration. Immunoblot analysis showed that p62 was upregulated in the optic nerve after intravitreal injection of TNF. Treatment with p62 small interfering RNA (siRNA) exerted a partial but significant protective effect against TNF-induced axonal loss. Rapamycin exerted substantial axonal protection after TNF injection. We found that the increase in p62 was significantly inhibited by p62 siRNA. Treatment with rapamycin also significantly inhibited increased p62 protein levels induced by TNF. These results suggest that the upregulation of p62 may be involved in TNF-induced axonal degeneration and that decreased p62 levels may lead to axonal protection.

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1. Introduction

Autophagy is involved in a cell-protective process and plays a role in cell death [1]. p62, which is also called sequestosome 1 (SQSTM1), plays central roles in the autophagy machinery in several types of cells. The accumulation of p62 has been linked to neurodegenerative disease [2–4]. A recent study has demonstrated that the overexpression of p62 promotes apoptosis with the activation of caspase-8, while knockdown of p62 reduces human glioma cell death [5]. p62 is normally degraded by the lysosomal proteases through the interaction with LC3-II, an autophagic marker [6]. In addition, a previous study demonstrated that p62 was upregulated in the compressed spinal cord and that the forced expression of p62 decreased the number of neuronal cells under hypoxic stress [7]. These findings imply that p62 plays a critical role in neuronal cell death.

The optic nerve consists of axons of retinal ganglion cells (RGCs), and transient increases in LC3-II are seen in RGCs after optic nerve transection [8]. Autophagy was reported to play a cytoprotective role in RGCs after traumatic injury [9]. In contrast, the inhibition of autophagy resulted in an attenuation of RGC death in a hypertensive glaucoma model [10]. Thus, the role of autophagy in axonal degeneration of RGCs remains to be examined. We previously demonstrated that there was a substantial increase in p62 protein levels in optic nerve samples 1 week after intraocular pressure (IOP) elevation in a rat hypertensive glaucoma model [11]. However, the role of p62 in axonal degeneration remains unclear. We therefore attempted to determine whether the modulation of

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p62 expression affects axonal loss in tumor necrosis factor (TNF)-induced optic nerve degeneration. TNF is involved in certain types of glaucoma [12–17], and the TNF injection model may be useful in understanding the mechanism of axonal degeneration of RGCs [18].

2. Materials and methods

2.1. Animals

Experiments were performed on 50- to 55-day-old male Wistar rats. All studies were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Ethics Committee of the Institute of Experimental Animals of St. Marianna University Graduate School of Medicine. The animals were housed in controlled conditions, with temperature at 23 ± 1 °C, humidity at 55 ± 5%, and light from 06:00 to 18:00.

2.2. Administration of TNF

Intravitreal injection of TNF (Sigma–Aldrich, St. Louis, MO, USA) was performed as described previously [18,19]. Briefly, rats were anesthetized with an intramuscular injection of a mixture of ketamine–xylazine (10 and 4 mg/kg, respectively). A single 2-μl injection of 10 ng TNF in 0.01 M PBS, pH 7.40, was administered intravitreally into the right eye of an animal under a microscope to avoid lens injury. PBS alone was administered into the contralateral left eye as a control. For p62 small interfering RNA (siRNA) treatment, 50 pmol of p62 siRNA (SQSTM1/p62 siRNA II #6399S, Cell Signaling Technology, Danvers, MA, USA) in 0.01 M PBS was administered intravitreally simultaneously mixed with a 10-ng TNF injection. In the rapamycin treatment group, 2 nmol of rapamycin (LC Laboratories, Woburn, MA, USA) in 95% dimethyl sulfoxide (DMSO)/dH_2O was administered intravitreally simultaneously mixed with a 10-ng TNF injection. A previous study used a single 1-μl intravitreal injection of 100% DMSO as sham control in mice and showed no RGC death [20]. Therefore, our current DMSO amount (2-μl intravitreal injection of 95% DMSO/dH_2O) in rats is likely to yield a similar final concentration. The rats were euthanized 1 or 2 weeks after the intravitreal injections with an intraperitoneal overdose of sodium pentobarbital, followed by enucleation of the eye.

2.3. Immunoblot analysis

Twenty-four rats were used for immunoblot analysis as described previously [21]. Briefly, 1 week after intravitreal injection, optic nerves (4 mm in length starting immediately behind the globe) were collected, homogenized, and then centrifuged at 15,000 × *g* for 15 min at 4 °C Two optic nerve specimens were pooled into one sample. Protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). Protein samples (5 μg per lane) were subjected to SDS-PAGE on gels (Bio-Rad) and transferred to PVDF membranes (Immobilon-P, Millipore, Billerica, MA, USA). Membranes were blocked with Tris-buffered saline (TBS)–0.1% Tween-20 containing 5% skim milk. Membranes were first reacted with anti-p62 antibody (1:200; Medical & Biological Laboratories Co., Nagoya, Japan) or anti-β-actin antibody (1:500; Sigma–Aldrich) in TBS containing 5% skim milk. Membranes were then sequentially exposed to peroxidase-labeled anti-rabbit IgG antibody (Cappel, Solon, OH, USA) or peroxidase-labeled anti-mouse IgG antibody (Cappel) diluted 1:5000 in Tween-20 in TBS. Western blots were visualized with an ECL detection system (Amersham ECL Prime Western Blotting Detection Reagents, GE Healthcare, Buckinghamshire, UK).

2.4. Axon counting in optic nerves

Morphometric analysis of each optic nerve was performed as described previously with samples from 18 rats [18,19,21]. Eyes were obtained from the animals 2 weeks after intravitreal injection. Four-millimeter segments of the optic nerves were obtained starting 1 mm behind the globe. These segments of optic nerve were fixed by immersion in Karnovsky's solution for 24 h at 4○C, processed, and embedded in acrylic resin. Cross sections (1 μm thick) were cut beginning 1 mm from the globe and stained with a solution of 1% paraphenylene–diamine (Sigma–Aldrich) in absolute methanol. For each section, images at the center and at each quadrant of the periphery (approximately 141.4 μm from the center) were acquired with a light microscope (BX51; Olympus, Tokyo, JAPAN) with a 100× coupled digital camera (MP5Mc/OL; Olympus) and associated QCapture Pro software (version 5.1, QImaging, Surrey, Canada). The acquired images were quantified using Aphelion image processing software (version 3.2, ADCIS SA and AAI, Inc., Hérouville Saint Clair, France). The number of axons was determined in five distinct areas of 1446.5 μm^2 each (each quadrant of the periphery in addition to the center; total area of 7232.3 μm^2 per eye) from each eye. The number of axons per eye was averaged and expressed as the number per square millimeter. A minimum of five eyes per experimental condition was used for analysis.

2.5. Statistical analysis

Data are presented as mean ± SEM. Differences among groups were analyzed using one-way ANOVA, followed by Scheffe's method or Mann–Whitney's method. A probability value of less

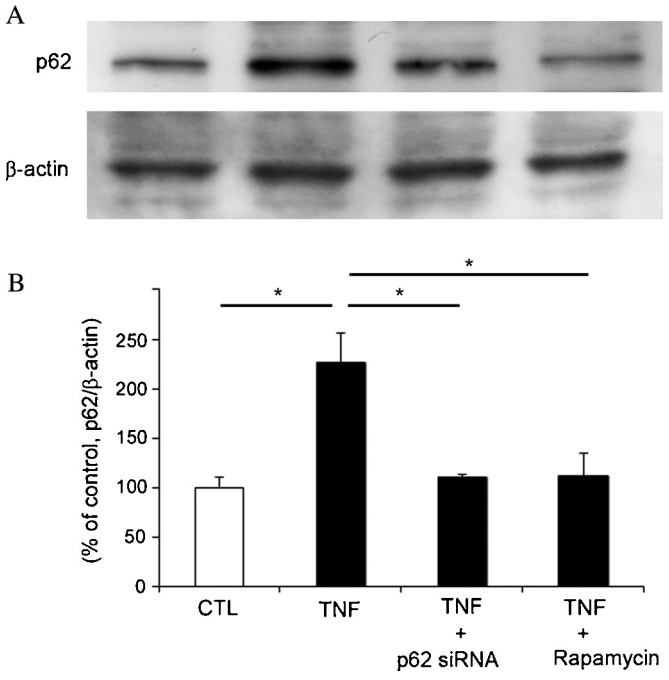


Fig. 1. p62 protein levels in optic nerves. Immunoblot data are normalized to β-actin levels in the same sample. (A) Immunoblotting for p62 1 week after intravitreal injection. (B) Data are expressed as a percentage of control. Each column represents mean ± SEM. $n = 4$ (eight optic nerves) per group. $^*p < 0.05$.

than 0.05 was considered to represent a statistically significant difference.

3. Results

3.1. Effects of TNF, p62 siRNA, and rapamycin on p62 protein levels in optic nerves

Because we previously found that p62 was increased in optic nerve samples 1 week after IOP elevation [11], in the current study we examined the changes in p62 protein levels in the optic nerve after intravitreal injection. We previously found that axonal loss started 1 week after TNF injection [18] and the molecular events before axon loss becomes obvious are important for clarifying the mechanism of axonal degeneration. There was a substantial increase in p62 protein levels in the optic nerve samples 1 week after TNF injection (Fig. 1). This increase was significantly inhibited by p62 siRNA (Fig. 1). Treatment with rapamycin also significantly inhibited the increase in p62 protein levels induced by TNF (Fig. 1).

3.2. Effects of modulation of p62 expression on TNF-induced axonal degeneration

Compared with PBS-treated eyes (Fig. 2A), we confirmed substantial degenerative changes in the axons 2 weeks after TNF injection (Fig. 2B), which is consistent with the findings of our previous studies [18,19,21]. p62 siRNA-treated eyes showed modest attenuated effects with better-preserved nerve fibers (Fig. 2C). Consistent with the findings that rapamycin exerted axonal protection in the hypertensive glaucoma model [11], treatment with rapamycin also showed substantial protective effects on axons against TNF-induced optic nerve degeneration (Fig. 2D). Quantitative analysis confirmed that axonal loss was 43% after TNF injection compared with controls (Fig. 2E). Treatment with p62 siRNA exerted a partial (38.8% protection) but significant protective effect against TNF-induced axonal loss ($n = 5$; $p < 0.05$ versus TNF injection; Fig. 2E). Rapamycin-treated eyes showed 76.3% axonal protection compared with eyes after TNF injection alone ($n = 5$; $p < 0.005$ versus TNF injection; Fig. 2E).

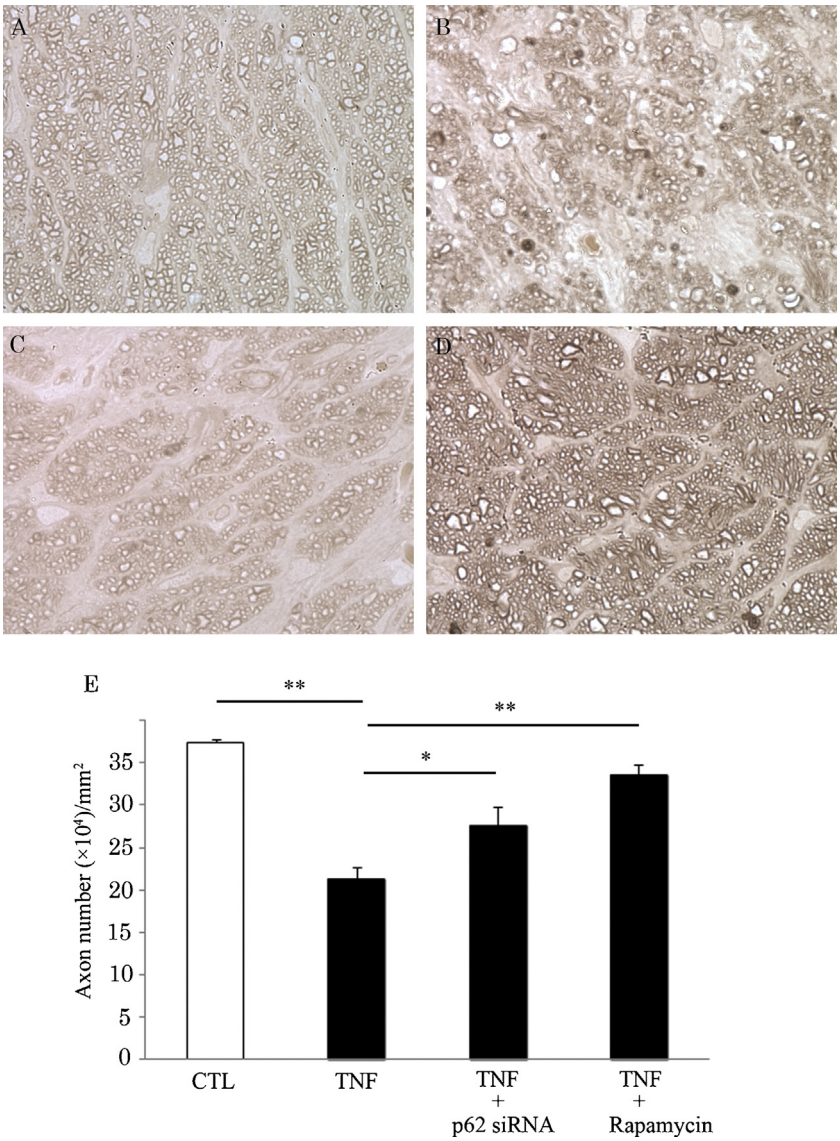


Fig. 2. Inhibition of p62 prevented TNF-induced axon loss. Light microscopic findings 2 weeks after (A) PBS injection, (B) 10 ng TNF injection, (C) 10 ng TNF + p62 siRNA injection, or (D) 10 ng TNF + rapamycin injection. Scale bar = 10 μm (A–D). (E) Effect of p62 siRNA or rapamycin on axon numbers in the optic nerve. Each column represents mean ± SEM; $n = 5$–8 per group. $^{*}p < 0.05$; $^{**}p < 0.005$.

4. Discussion

The mammalian target of rapamycin (mTOR) activity was suppressed and new protein synthesis was impaired in axotomized RGCs, which may contribute to the failure to regenerate [22]. Since rapamycin is an inhibitor of mTOR, it does not seem beneficial for axonal regeneration. However, a recent study has shown that mTOR activity is not generally required for neuroprotection or switching mature neurons into an active regenerative state in adult RGCs [23]. In the present study, rapamycin, an autophagy inducer, exerted a substantial protective effect against axonal loss after TNF injection. This finding is consistent with our previous results demonstrating that rapamycin exerted a significant protective effect against axonal loss after IOP elevation [11] and with those of another study demonstrating that decreased Brn-3a-immunopositive RGCs in flat-mounted retinas after optic nerve transection were significantly increased by rapamycin [9]. Thus, it is possible that rapamycin can exert protective effects against several distinct optic nerve injury models, although a discrepancy was found between neuroprotection and axonal regeneration [24].

In the present study, there was increase in p62 in the optic nerve after TNF injection. It was demonstrated that under pathological conditions, there is a constitutively high level of p62, thereby leading to the accumulation of damaged mitochondria and subsequent reactive oxygen species production [25]. We cannot exclude the possibility that TNF-induced oxidative stress itself could upregulate p62, since a previous report demonstrated that lipopolysaccharide mediated transcriptional up-regulation of p62 in RAW cells [26]. It was also demonstrated that the accumulation of p62 after autophagy inhibition caused a delay in the clearance of short-lived ubiquitin–proteasome system-specific substrates, like p53, which may mediate toxicity [27]. Since p62 accumulates when autophagy is inhibited, and decreased levels can be observed when autophagy is induced, p62 may be used as a marker of autophagy flux [28]. Another aspect is that the level of p62 may be dependent on the balance of incoming, which is the transcriptional regulation in response to various stimuli, and outgoing flux, which is influenced by autophagic activity [29]. Although we could not determine whether autophagic flux was impaired in the optic nerve in this study, the finding of an increase in p62 protein levels after TNF injection is consistent with that of a previous study demonstrating that a sublethal dose of TNF significantly increased the level of p62 protein in PC12 cells [30]. That study suggested that TNF acts not only as an inflammation mediator but also as a trigger of autophagic impairment and neuronal damage [30]. It is reasonable to speculate that p62 siRNA inhibited the neosynthesis of p62 protein in response to TNF injection. Another possibility is that p62 siRNA may affect autophagy activity, because a recent study has demonstrated that p62 siRNA activated autophagic machinery as confirmed by increases in lysosome-associated membrane protein 1, LC3-II, and beclin 1 and by the formation of autophagosomes [31]. Therefore, it is possible that p62 siRNA exerts axonal protection with the involvement of autophagy machinery. However, the inhibition of p62 alone exerted partial axonal protection. A recent report has suggested that p62 is a signaling node for multiple pathways and can potentially foster interaction between such pathways in order to maintain cellular homeostasis [32], implicating that p62 has distinct roles depending on the physiological or pathological conditions, or the status of proteasome activity. It was shown that autophagy activation increased LC3-II levels and decreased p62 levels in several types of neuron [7,33]. In addition, we also found that rapamycin completely abolished the increase in p62 levels in the optic nerve induced by TNF. The greater protective effect of rapamycin may be because it not only inhibits p62 but also has other effects such as increasing in autophagic flux. Nonetheless, taking the results together, upregulation of p62 may be involved in TNF-induced axonal degeneration and decreased p62 levels may lead to axonal protection.

In conclusion, the present study showed that modulation of p62 levels in the optic nerve may affect axonal degeneration. Clarification of the detailed mechanism of the regulation of p62 levels may lead to a new strategy for axonal protection in certain types of optic neuropathy.

Conflict of interest

The authors have no conflict of interest to disclose.

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