



Axonal Protection by Nicotinamide Riboside via SIRT1-Autophagy Pathway in TNF-Induced Optic Nerve Degeneration

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Abstract

Nicotinamide adenine dinucleotide (NAD⁺) synthesis pathway has been involved in many biological functions. Nicotinamide riboside (NR) is widely used as an NAD⁺ precursor and known to increase NAD⁺ level in several tissues. The present study aimed to examine the effect of NR on tumor necrosis factor (TNF)-induced optic nerve degeneration and to investigate whether it alters SIRT1 expression and autophagic status in optic nerve. We also examined the localization of nicotinamide riboside kinase 1 (NRK1), which is a downstream enzyme for NR biosynthesis pathway in retina and optic nerve. Intravitreal injection of TNF or TNF plus NR was performed on rats. The p62 and LC3-II protein levels were examined to evaluate autophagic flux in optic nerve. Immunohistochemical analysis was performed to localize NRK1 expression. Morphometric analysis showed substantial axonal protection by NR against TNF-induced axon loss. TNF-induced increment of p62 protein level was significantly inhibited by NR administration. NR administration alone significantly increased the LC3-II levels and reduced p62 levels compared with the basal levels, and upregulated SIRT1 levels in optic nerve. Immunohistochemical analysis showed that NRK1 exists in retinal ganglion cells (RGCs) and nerve fibers in retina and optic nerve. NR administration apparently upregulated NRK1 levels in the TNF-treated eyes as well as the control eyes. Pre-injection of an SIRT1 inhibitor resulted in a significant increase of p62 levels in the NR plus TNF treatment group, implicating that SIRT1 regulates autophagy status. In conclusion, NRK1 exists in RGCs and optic nerve axons. NR exerted protection against axon loss induced by TNF with possible involvement of upregulated NRK1 and SIRT1-autophagy pathway.

Keywords Nicotinamide riboside · NRK1 · Autophagy · SIRT1 · p62

Introduction

Nicotinamide adenine dinucleotide (NAD⁺) synthesis pathway has been involved in many biological functions. NAD⁺ is synthesized by salvage of vitamin precursors, nicotinic acid (NA), nicotinamide, and nicotinamide riboside (NR). Nicotinamide phosphoribosyltransferase (Nampt) converts nicotinamide into nicotinamide mononucleotide (NMN), whereas nicotinamide riboside kinase 1 (NRK1) converts NR into NMN. Then,

NMN is converted to NAD⁺ by nicotinamide nucleotide adenylyltransferase1–3 (Nmnat1–3) [1]. Nmnat1–3 are enzymes which have been reported to link to axonal protection in dorsal root ganglia [2–6]. Nmnat1–3 were found to exist in optic nerve [7–9], and Nmnat2 is required to retinal ganglion cell (RGC) axon growth [9]. It was reported that cytoplasmic overexpression of Nmnat1 protected against glaucomatous RGC axon loss [10]. Our previous study demonstrated that overexpression of Nmnat3 protected against glaucomatous RGC axon loss and tumor necrosis factor (TNF)-induced axon loss [8]. On the other hand, a previous study showed a decrease in NAD level in retina in DBA/2J mice [11]. This is agreement with our previous study showing a decrease in NAD level in optic nerve in TNF-induced axon damage model [7]. Interestingly, a recent study demonstrated a significantly lower plasma nicotinamide concentration in primary open-angle glaucoma patients compared with the control group [12]. Since oral intake of vitamin B3/nicotinamide increased NAD level in retina and exerted axonal protection in DBA/2 J mice [11], it is reasonable to postulate that the nicotinamide supplementation may have a beneficial effect for certain glaucomatous damages.

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NR is widely used as an NAD⁺ precursor supplementation and has been reported to increase the blood NAD⁺ level in humans [13]. NRK1 is the central rate-limiting enzyme in driving NAD⁺ synthesis from NR [14]. However, localizations of NRK1 in retina and optic nerve have not been documented. There have been several studies suggesting critical roles of NAD⁺ and its precursors on autophagy machinery [15–17]. Autophagy is a cellular process including the clearance of unnecessary proteins and maintains homeostasis in several types of neurons. We and others reported that autophagy plays crucial roles in certain different optic nerve damages such as optic nerve crush model, hypertensive glaucoma model, and TNF-induced axon damage model [18–21]. For autophagy research, SQSTM1/p62 is used as a marker and decrease of p62 level is associated with autophagy activation [22]. In yeast, NR upregulates NAD⁺ levels, enhances Sir2 functions, and extends lifespan [23]. Sirtuin 1 (SIRT1), the mammalian homolog of yeast Sir2, can be activated by calorie restriction [24], NAD⁺, and its precursors [25]. Thus, the purpose of present study is to examine the effect of NR on TNF-induced axonal degeneration and to investigate whether it alters SIRT1 expression and autophagic status in optic nerve. We also examined the localization of NRK1, which is a downstream enzyme for NR biosynthesis pathway in retina and optic nerve as well as the alteration of NRK1 expression. Finally, we tested if an inhibitor of SIRT1 alters autophagy status.

Materials and Methods

Animals

Experiments were carried out on 8-week-old male Wistar rats. All studies were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by Ethics Committee of the Institute of Experimental Animals of St. Marianna University School of Medicine. The animals were kept in the controlled rooms (23 ± 1 °C; humidity at 55 ± 5%; light from 6 a.m. to 6 p.m.).

Intravitreal Administrations

Intravitreal injection of TNF (Sigma-Aldrich, St. Lois, MO) was performed as described previously [7]. Phosphate-buffered saline (PBS) was used as a control. Anesthetization with intramuscular injections of a mixture of ketamine-xylazine was conducted. NR triflate was purchased from Toronto Research Chemicals

(North York, ON, Canada), dissolved in PBS. Concomitant injection of 2, 20, and 200 pmol of NR and 10 ng TNF was performed intravitreally. For immunoblotting, NR alone injection was also performed. For the SIRT1 inhibitor study, EX-527 (Sigma-Aldrich) was dissolved in DMSO and 200 pmol of EX-527 or DMSO alone was injected intravitreally 10 min before intravitreal injection of NR plus TNF. One and 2 weeks after intravitreal injection, the rats were euthanatized with overdose of sodium pentobarbital and the eyes were enucleated.

Immunoblotting

Optic nerve specimens (4-mm lengths) were gathered and homogenized in protein extraction buffer 1 week after injection. Homogenized samples were then centrifuged at 15,000×g for 15 min at 4 °C. Protein concentrations were determined with the supernatants. Each sample (3 μg) was applied and subjected to the mini gel (Bio-Rad Laboratories) and transferred to enhanced chemiluminescent membrane (EMD Millipore Corporation, Temecula, CA). The membranes were blocked with 5% skim milk with tris buffered saline (TBS) containing Tween-20 and reacted with anti-p62 antibody (MBL Life Science, Nagoya, Japan), anti-LC3 antibody (MBL Life Science), anti-SIRT1 antibody (Santa Cruz Biotechnology), anti-NRK1 antibody (Lifespan Biosciences Inc. Seattle, WA) or anti-β-actin antibody (Sigma-Aldrich). After three times washing, the membranes were reacted with anti-rabbit or anti-mouse peroxidase-labeled secondary antibody (MP Biochemicals, Solo, OH). Immunoblotting was visualized with a chemiluminescence detection system (ECL Plus Western Blotting Detection Reagents, Amersham Pharmacia Biotech).

Immunohistochemistry

Three eyes 1 week after intravitreal injection of NR or three normal eyes were collected and fixed by immersion in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections were made through the optic disc and blocked with 1% bovine serum (Roche Diagnostics GmbH, Mannheim, Germany). The primary antibodies were against NRK1 (1:100; LifeSpan BioSciences), neurofilament-L (a marker of nerve fibers; 1:100; Dako, Tokyo, Japan), or Thy-1 (a marker of RGC; 1:50; Santa Cruz Biotechnology, TX). The secondary antibodies were FITC-labeled or rhodamine-labeled antibodies (1:100; Cappel, Aurora, OH). The sections were mounted on slides in DAPI-containing medium with cover glass.

Quantification of Optic Nerve Axons

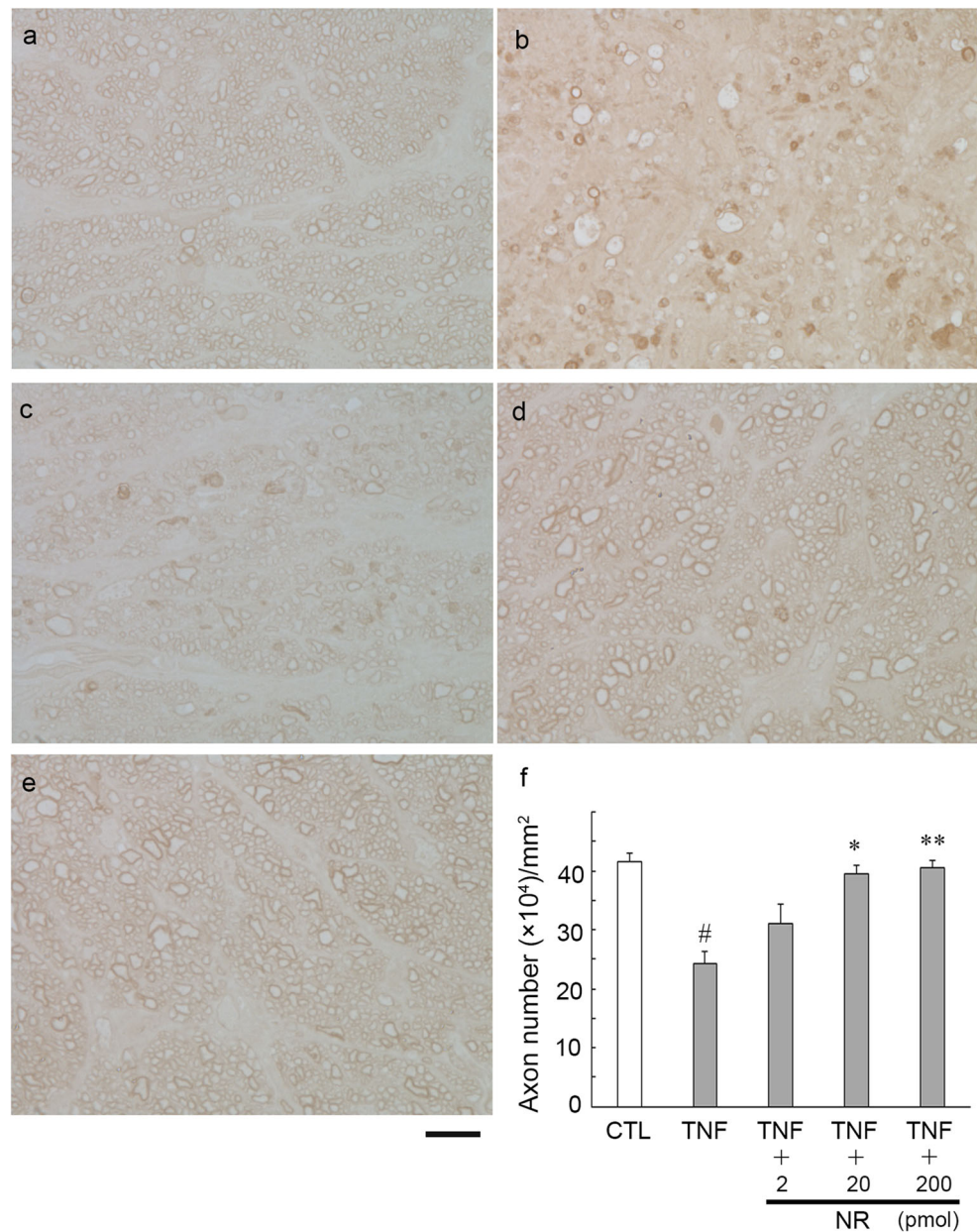
Optic nerve specimens (4-mm lengths from 1 mm behind the globe) were collected and soaked in Karnovsky's solution for 24 h at 4 °C 2 weeks after injection. Several dehydrations were performed, and samples were embedded in acrylic resin at 70 °C two overnight. Then, samples were sectioned and stained with 1% paraphenylene-diamine (Sigma-Aldrich) in absolute methanol [7, 26]. This can stain myelin, and five black and white images from each eye were obtained at the center and at each quadrant of the periphery with a light microscope (Olympus, Tokyo, Japan). These black and white images (each area is 5850 μm^2 , and total area is 29,250 μm^2 per eye) were used for quantification with the Aphelion image

processing software (ADCIS S.A., Hérouville Saint-Clair, France). The number of axons was averaged in each eye and each group, and data were presented as the number per square millimeter. After quantification, representative color photos were obtained.

Statistical Analysis

Data are expressed as mean \pm SEM. Differences among groups were analyzed by one-way ANOVA with post-hoc Tukey's HSD test or Mann–Whitney method. A probability value was considered statistically significant when $p < 0.05$.

Fig. 1 Paraphenylene-diamine staining of optic nerve axons 2 weeks after injection. **a** Control group. **b** TNF-injected group. **c** 2 pmol NR + TNF-injected group. **d** 20 pmol NR + TNF-injected group. **e** 200 pmol NR + TNF-injected group. Scale bar = 10 μm . **(f)** Morphometric analysis of axon number. (CTL: $n = 5$, TNF: $n = 5$, 2 pmol NR + TNF: $n = 6$, 20 pmol NR + TNF: $n = 7$, 200 pmol NR + TNF: $n = 7$) ($P^\# < 0.0001$ vs. CTL, $P^* < 0.0005$ vs. TNF, $P^{**} < 0.0001$ vs. TNF)



Results

Effects of NR on TNF-Induced Axon Loss in Optic Nerve

As shown previously [7], histological findings again showed substantial degenerative changes and apparent axon losses after TNF injection (Fig. 1b) compared with the control (Fig. 1a). Co-injection with 2 pmol NR plus TNF showed slightly protective tendency (Fig. 1c). However, this was not statistically significant ($p = 0.1850$ vs. TNF; Fig. 1f). Co-injection with 20 or 200 pmol NR plus TNF showed noticeable protective effects compared with the TNF alone injection (Fig. 1d and e, respectively). The quantitative analysis showed remarkable protective effects against TNF-induced axon loss, and these were statistically significant (20 pmol and 200 pmol NR: $p = 0.0002$ vs. TNF, and $p < 0.0001$ vs. TNF, respectively; Fig. 1f).

Effects of TNF and NR on LC3-II Protein Levels in Optic Nerve

There was a significant increase in the LC3-II level in the treatment with 200 pmol NR plus TNF as compared with those in the treatment with TNF at 1 week (Fig. 2a). Moreover, 200 pmol NR alone administration significantly increased the LC3-II level compared with the control group (Fig. 2b).

Effects of TNF and NR on p62 Protein Levels in Optic Nerve

In agreement with our previous findings [27], p62 protein level was significantly increased in optic nerve in TNF-treated group at 1 week (Fig. 3a). Treatment with 200 pmol NR plus TNF completely prevented this increase of p62 (Fig. 3a). In addition, 200 pmol NR alone administration significantly decreased p62 protein level compared with the control group (Fig. 3b).

Effects of TNF and NR on SIRT1 Protein Levels in Optic Nerve

As we recently found [28], no significant change in SIRT1 protein level was seen in between TNF-treated group and PBS-treated group (Fig. 4a). However, treatment with 200 pmol NR plus TNF significantly increased the SIRT1 levels compared with TNF alone treatment (Fig. 4a). Moreover, 200 pmol NR alone administration significantly upregulated the SIRT1 levels compared with the control group (Fig. 4b).

NRK1 in Retina and Optic Nerve

To examine the effect of NR and its metabolic pathway further, we investigated the localization of NRK1 in the retina and optic nerve. In the normal retina, the NRK1 immunoreactive pattern was similar to that of Thy-1 immunoreactivity (Fig. 5, upper panels). Most NRK1-positive cells were colocalized with Thy-1-positive cells (Fig. 5, upper panels). The NRK1 immunoreactivity was also observed in the nerve fiber layer, and these were

Fig. 2 Immunoblotting in samples from optic nerves 1 week after injection. a Effects of TNF or 200 pmol NR + TNF on LC3-II protein level (CTL: $n = 3$, TNF: $n = 3$, TNF + NR: $n = 3$) ($P^{**} < 0.005$). (b) Effects of 200 pmol NR on LC3-II protein level. (CTL: $n = 3$, NR: $n = 3$) ($P^* < 0.05$)

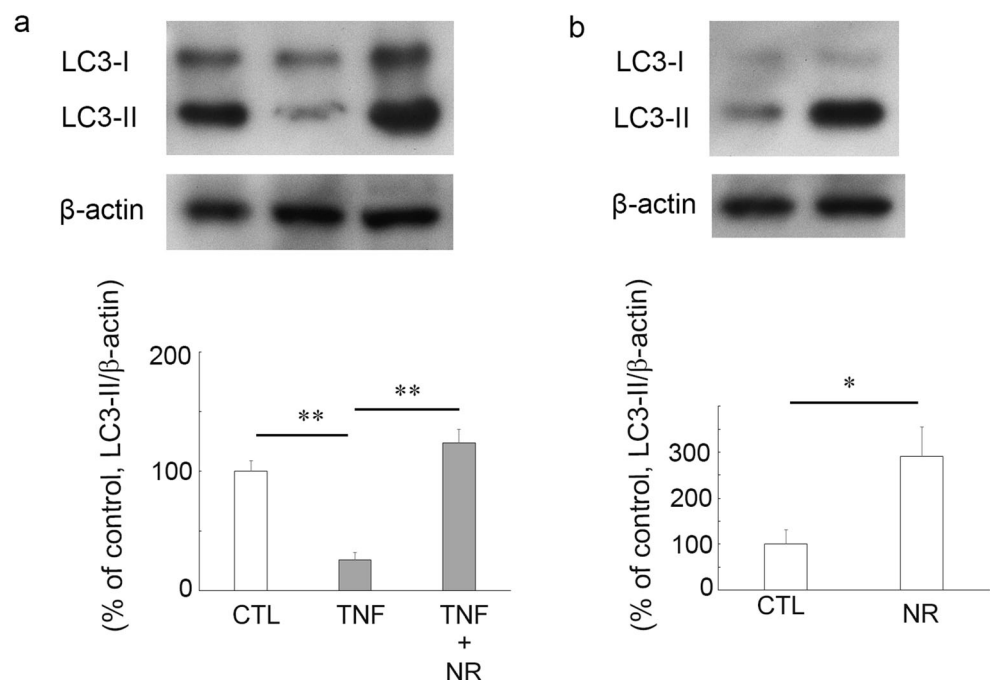
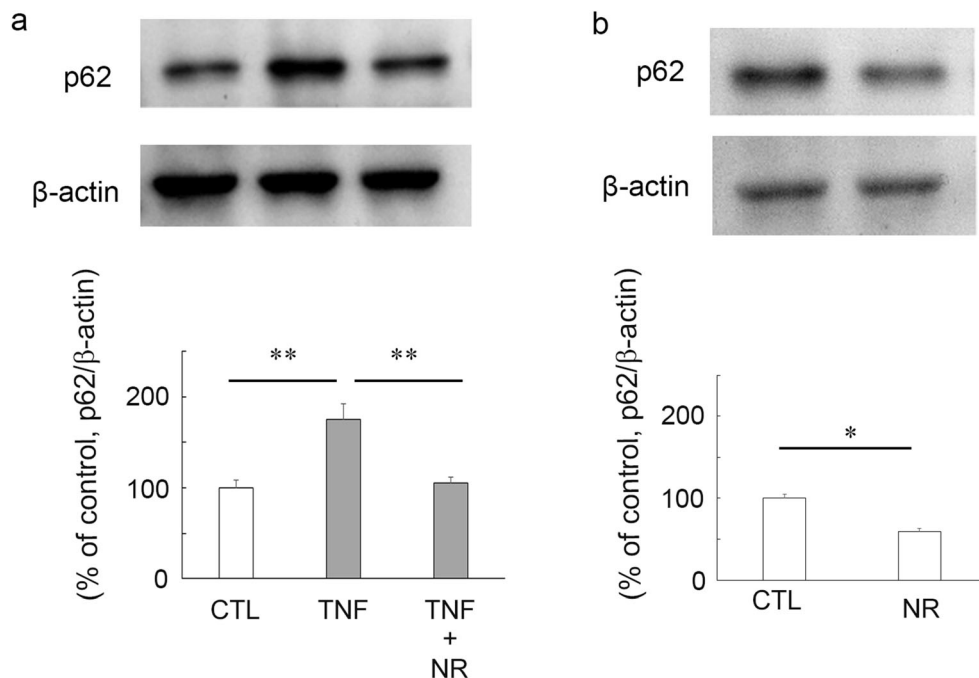


Fig. 3 Immunoblotting in samples from optic nerves 1 week after injection. **a** Effects of TNF or 200 pmol NR + TNF on p62 protein level. (CTL: $n = 4$, TNF: $n = 4$, TNF + NR: $n = 4$) ($P^{**} < 0.005$). **b** Effects of 200 pmol NR on p62 protein level. (CTL: $n = 5$, NR: $n = 5$) ($P^* < 0.05$)

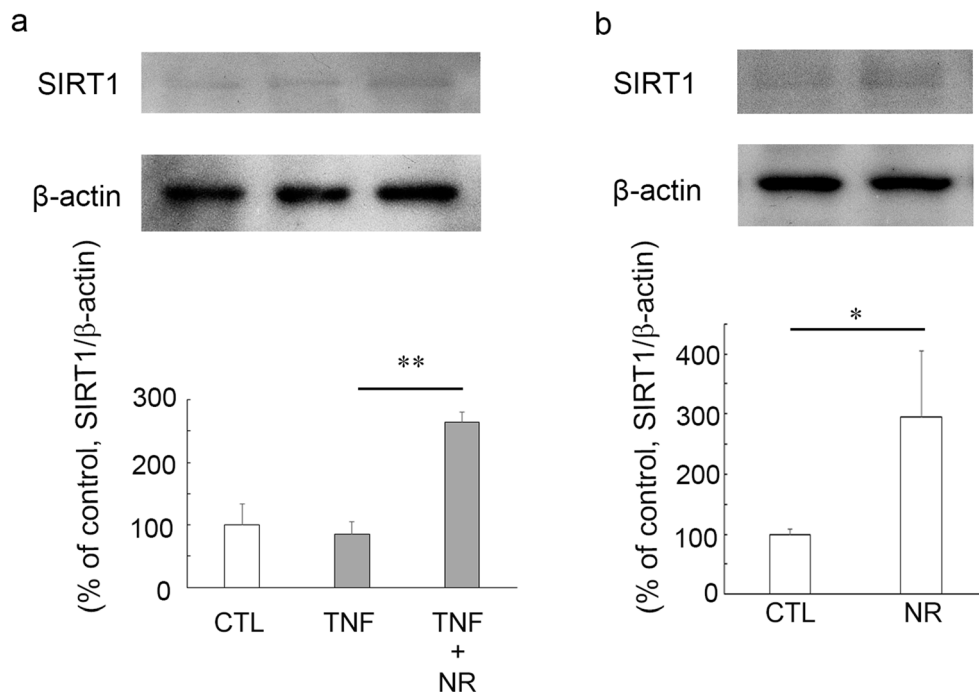


colocalized with neurofilament immunoreactivity (Fig. 5, upper middle panel). In the optic nerve, the immunoreactivity of NRK1 was modest, but some immunopositive fibers were colocalized with neurofilament immunoreactivity (Fig. 5, lower middle panel). In the optic nerve after NR treatment, a lot of NRK1 immunopositive fibers were apparently colocalized with neurofilament immunoreactivity (Fig. 5, lower panel). In the retina after NR treatment, similar findings to the normal eyes were observed (Suppl. Fig. 1).

Effects of TNF and NR on NRK1 Protein Levels in Optic Nerve

We next investigated the change in NRK1 expression in optic nerve. There was a tendency of decrease in NRK1 protein levels after TNF injection (Fig. 6a). Unexpectedly, treatment with 200 pmol NR plus TNF significantly increased the NRK1 levels compared with TNF alone treatment (Fig. 6a). Furthermore, 200 pmol NR alone administration

Fig. 4 Immunoblotting in samples from optic nerves 1 week after injection. **a** Effects of TNF or 200 pmol NR + TNF on SIRT1 protein level. (CTL: $n = 4$, TNF: $n = 4$, TNF + NR: $n = 4$) ($P^{**} < 0.005$). **b** Effects of 200 pmol NR on SIRT1 protein level. (CTL: $n = 3$, NR: $n = 3$) ($P^* < 0.05$)



significantly upregulated the NRK1 levels compared with the control group (Fig. 6b).

Effects of an SIRT1 Inhibitor on p62 Protein Level in the NR plus TNF Treatment in Optic Nerve

To investigate whether an inhibitor of SIRT1 alters autophagy status, pre-injection of EX-527 was performed before co-injection with 200 pmol NR and TNF. Pre-injection of EX-527, an inhibitor of SIRT1, significantly upregulated p62 levels as compared with NR plus TNF treatment group (Fig. 7).

Discussion

The present study revealed that intravitreal injection of NR exerted substantial axonal protection in TNF-induced optic nerve degeneration. Recent studies have demonstrated beneficial effects of NR on central nervous system. For example, NR treatment prevented dopaminergic neuronal loss in Parkinson's disease model flies [29]. Moreover, NR treatment ameliorated selective cognitive impairment in aged mice and decreased the number of amyloid beta plaques in cortex of Alzheimer's disease model mice [30]. Furthermore, NR treatment decreased glial activation and delayed motor neuron loss

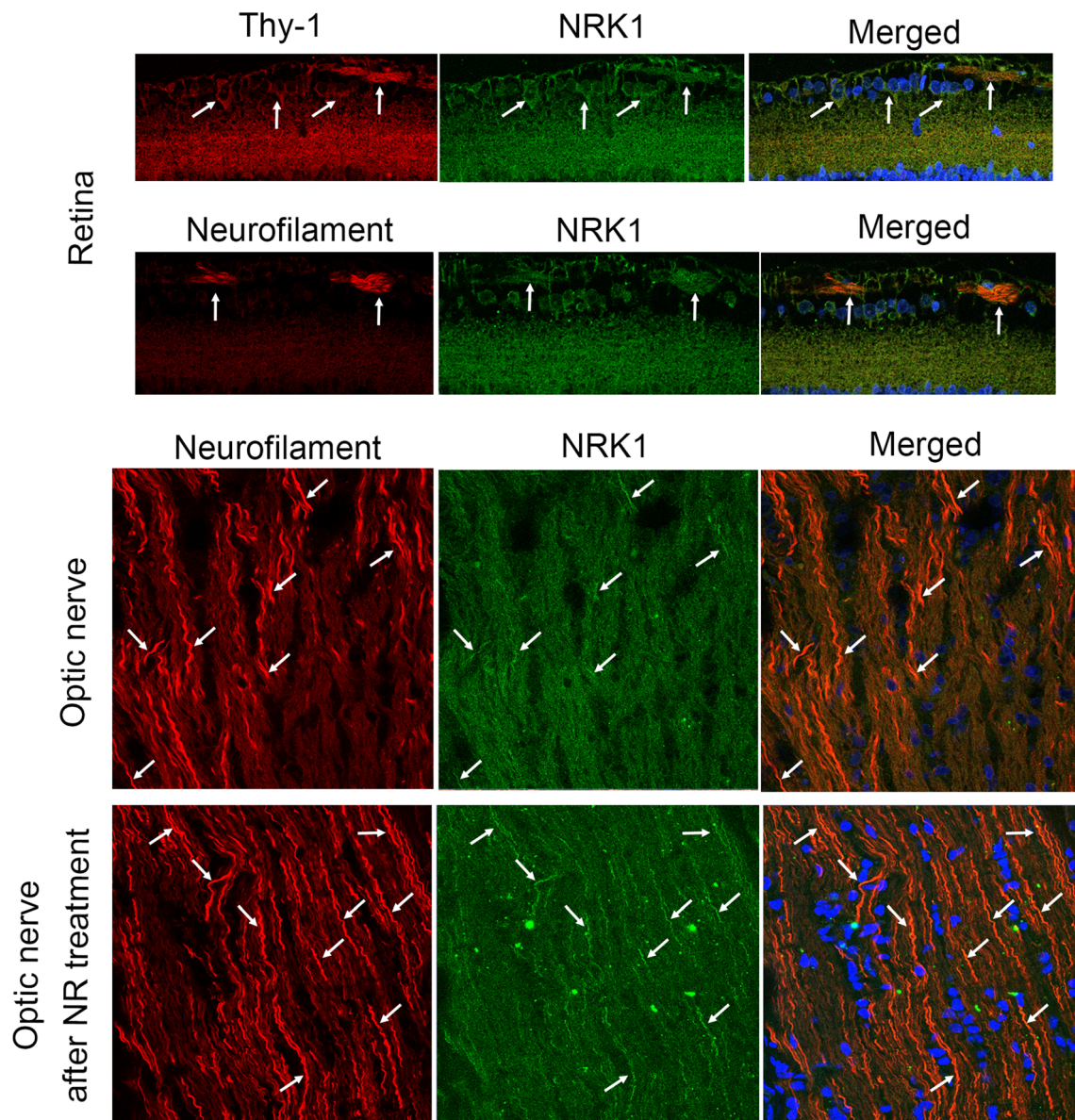
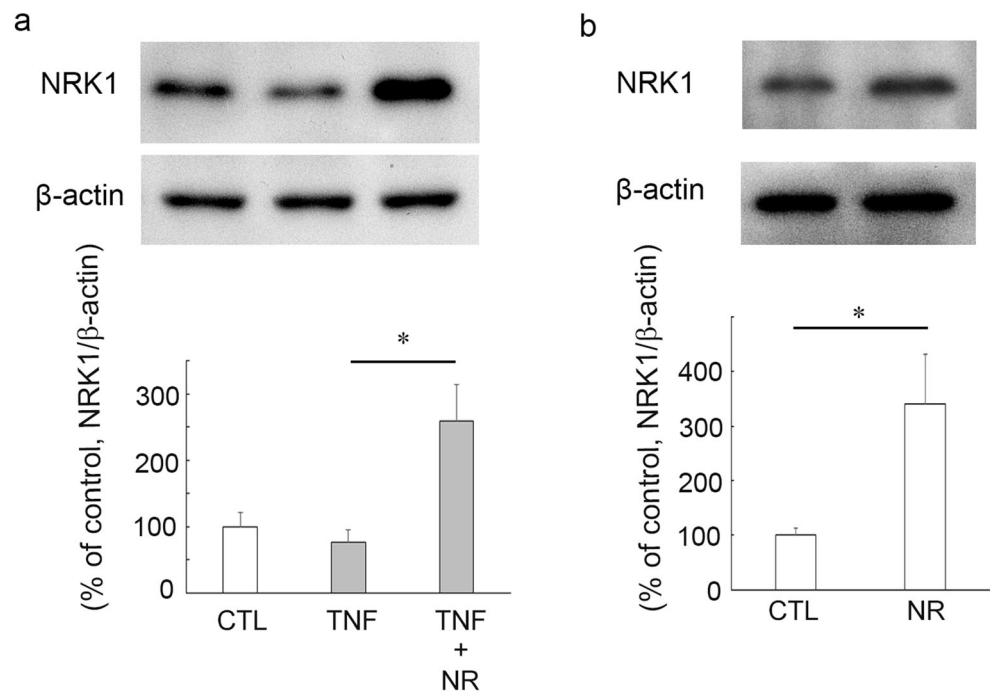


Fig. 5 Immunohistochemistry in retina and optic nerve. NRK1-positive cells were colocalized with Thy-1-positive cells in normal retina. NRK1 immunoreactivity was colocalized with neurofilament immunoreactivity in normal retina. A few NRK1 immunoreactivities were colocalized with

neurofilament immunoreactivity in normal optic nerve, but a lot of NRK1 immunopositive fibers were colocalized with neurofilament immunoreactivity in the NR-treated optic nerve. Arrows indicate colocalization. Scale bar = 50 μ m

Fig. 6 Immunoblotting in samples from optic nerves 1 week after injection. **a** Effects of TNF or 200 pmol NR + TNF on NRK1 protein level. (CTL: $n = 4$, TNF: $n = 4$, TNF + NR: $n = 4$) ($P^* < 0.05$). **b** Effects of 200 pmol NR on NRK1 protein level. (CTL: $n = 3$, NR: $n = 3$) ($P^* < 0.05$)



in the spinal code of amyotrophic lateral sclerosis model mice [31]. In axons, it was previously reported that NR significantly delayed axonal degeneration in dorsal root ganglia neurons [5]. More recently, it was shown that NR prevented axonal degeneration induced by excitotoxicity in cortical neurons [32]. Collectively, NR may have a protective effect on several types of axons against distinct injury models.

Our previous study suggested that upregulated p62 levels indicate impairment of autophagic flux in optic nerve [8, 18, 27]. In the present study, upregulated p62 levels induced by TNF were significantly prevented by NR. NR significantly increased LC3-II protein levels in both the TNF-treated group and the control group. NR also significantly reduced p62 protein level compared with the basal level, implicating that NR can enhance autophagic flux. It is worthy to note that nicotinamide protects against palmitate-induced hepatotoxicity through SIRT1-dependent autophagy induction [33]. Since SIRT1 activators stimulate the autophagy [34, 35] and increased NAD^+ stimulates SIRT1 activity [36], we speculated that this pathway may exist between NR and autophagy induction. Therefore, we further examined SIRT1 expression and found that NR upregulated SIRT1 protein level in optic nerve. Consistently, a very recent study demonstrated that treatment of NR upregulated SIRT1 activity and decreased neuroinflammation in the brains of Gulf War Illness mice [37], suggesting that NR can activate SIRT1 in certain neuronal system as well as in optic nerve. Moreover, our recent study demonstrated that an SIRT1 activator exerted axonal protection with upregulated autophagic status [28]. Furthermore, the current study found that the SIRT1 inhibitor significantly upregulated p62 level in the NR plus TNF treated

group, implicating that inhibition of SIRT1 leads to autophagy impairment. Taken together, these findings suggest that NR attenuated axonal degeneration via SIRT1-autophagy

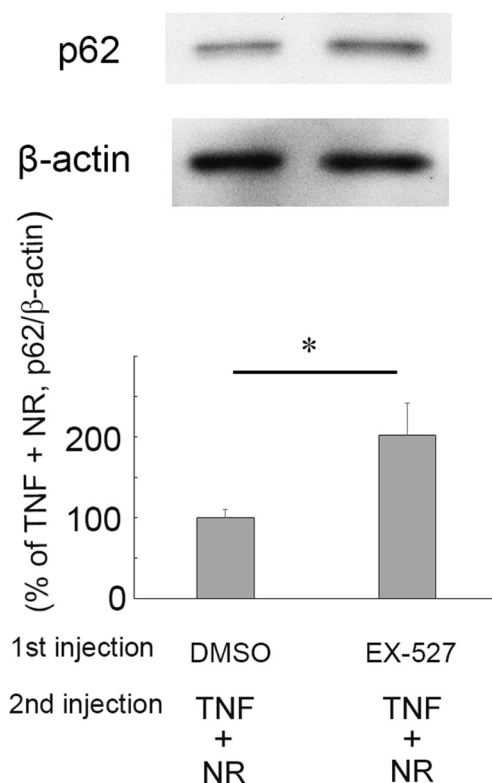


Fig. 7 Immunoblotting in samples from optic nerves 1 week after injection. Effects of EX-527 on p62 protein level in the 200 pmol NR plus TNF-treated group. (pre-injection of DMSO and TNF + NR: $n = 4$, pre-injection of EX-527 and TNF + NR: $n = 4$) ($P^* < 0.05$)

pathway. In line with this concept, a previous study demonstrated the neuroprotective activity of cilostazol via SIRT1-autophagy activation in rat Parkinson's disease model [38].

Although Nmnats were found in optic nerve [7–9], NRK1 has not been examined in retina and optic nerve. Thus, the present study firstly showed that NRK1 exists in RGCs and optic nerve axons. This finding makes it possible that locally applied exogenous NR can accelerate NAD biosynthesis and activate downstream effectors. Surprisingly, NR administration clearly boosted NRK1 levels in the TNF-treated eyes as well as the control eyes. The mechanism of this regulation is unclear, while the regulation of NRK2 in muscle has been proposed in response to various conditions [39]. One hypothesis posits that exogenous NR may recruit more NRK1, thereby accelerating conversion to downstream effectors.

In conclusion, NR exerts axonal protection against TNF-induced optic nerve degeneration with the possible upregulated NRK1 and through SIRT1-autophagy pathway.

Authors' Contributions YK designed the whole study, performed the intravitreal injection, enucleated eyes, performed immunohistochemistry, and wrote the manuscript. KS performed axon counting and immunoblot analysis and interpreted data. CT performed the protein assay and prepared several samples for immunoblot and immunohistochemistry. NF performed the process for plastic sections and interpreted data. IA prepared several samples for immunoblot and immunohistochemistry. HT performed statistical analysis and wrote the manuscript. All authors have read and approved the manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

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