Corneal Denervation Causes Epithelial Apoptosis Through Inhibiting NAD⁺ Biosynthesis

Ya Li,1,2 Xiubin Ma,1 Jing Li,3 Lingling Yang,2 Xiaowen Zhao,2 Xia Qi,2 Xiaoping Zhang,1 Qingjun Zhou,2 and Weiyun Shi2

1Medical College, Qingdao University, Qingdao, China
2State Key Laboratory Cultivation Base, Shandong Provincial Key Laboratory of Ophthalmology, Shandong Eye Institute, Shandong First Medical University & Shandong Academy of Medical Sciences, Qingdao, China
3Medical College, University of Jinan, Jinan, China

Correspondence: Weiyun Shi, State Key Laboratory Cultivation Base, Shandong Provincial Key Laboratory of Ophthalmology, Shandong Eye Institute, Shandong First Medical University & Shandong Academy of Medical Sciences, 5 Yan’erdao Road, Qingdao 266071, China; weiyunshi@163.com. Qingjun Zhou, State Key Laboratory Cultivation Base, Shandong Provincial Key Laboratory of Ophthalmology, Shandong Eye Institute, Shandong First Medical University & Shandong Academy of Medical Sciences, 5 Yan’erdao Road, Qingdao 266071, China; qijzhou2000@hotmail.com.

Submitted: February 19, 2019
Accepted: July 22, 2019

PURPOSE. To determine if trigeminal innervations of the corneal epithelium maintains its integrity and homeostasis through controlling the nicotinamide adenine dinucleotide (NAD) content of this tissue.

METHODS. Corneal denervation of C57BL/6 mice was induced by squeezing the nerve bundles that derive from the trigeminal ganglion and was confirmed by whole-mount corneal nerve staining and the sensation test. The apoptosis of the corneal epithelium was examined by TUNEL assay and annexin V/propidium iodide staining. NAD biosynthesis-related enzymes were analyzed by quantitative PCR, immunofluorescence staining, and Western blotting. FK866, an inhibitor of nicotinamide phosphoribosyltransferase (NAMPT), exogenous nicotinamide mononucleotide (NMN), and NAD⁺ were used to evaluate the effect of NAD⁺ on the apoptosis of cultured corneal epithelial cells and epithelial detachment in denervated mice. Protein expression that related to apoptosis and phosphorylation were analyzed by Western blotting.

RESULTS. The denervated mice showed spontaneous corneal epithelial detachment and cell apoptosis accompanied by impaired epithelial NAD⁺ contents due to low levels of NAMPT. Similarly, inhibition of NAMPT recapitulated epithelial detachment as in denervated mice and induced apoptosis in cultured corneal epithelial cells. The replenishment of NMN or NAD⁺ partially slowed down corneal nerve fiber degeneration, reduced the epithelial defect in denervated mice, and improved apoptosis induction in FK866-treated cells by restoring the activation levels of SIRT1, AKT, and CREB.

CONCLUSIONS. Corneal denervation lowered epithelial NAD⁺ contents through reducing the expression of NAMPT and caused cell apoptosis and epithelial defects, suggesting that corneal innervations contribute to epithelial homeostasis by regulating NAD⁺ biosynthesis.

Keywords: corneal denervation, corneal epithelium, apoptosis, NAMPT, NAD⁺, NMN
cardiomyopathy, Alzheimer’s disease, and cancer. In the degeneration-related diseases of the retina, brain, liver, skeletal muscle, and cardiomyocytes, NAD$^+$ content is lower, while either NAMPT overexpression or replenishment with either NAD$^+$ or its precursors reversed declines in cell survival.

To determine how corneal epithelial denervation induces apoptosis, we measured the effects of this procedure on NAD$^+$ content and NAMPT expression level. The results showed that the corneal epithelial apoptosis induced by denervation was accompanied by low epithelial NAD$^+$ content and was due to low expression of NAMPT. The inhibition of NAD$^+$ biosynthesis by the NAMPT inhibitor recapitulated corneal denervation-induced epithelial detachment and cell apoptosis, which was partially improved by the replenishment of NAD$^+$ or NMN. Therefore, our results revealed that corneal denervation-induced epithelial apoptosis through the inhibition of NAD$^+$ biosynthesis.

**MATERIALS AND METHODS**

**Animal Models**

C57BL/6 mice (8-week-old males) purchased from the Pengyue Company (Jinan, Shandong, China) were used in this study. The animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The corneal denervation model was constructed as described in a previous study. Briefly, animals were systemically anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal) and topically anesthetized with 2% xylocaine. The nerve was exposed by cutting the conjunctiva at the lateral canthus of the mouse’s right eye, and the nerve bundles derived from trigeminal ganglion were separated. The nerve bundles were squeezed for 30 seconds using forceps, and the conjunctiva was sutured (Fig. 1A). The cornea of the surgical eye was frequently moistened with isotonic saline throughout the operation. The second day after surgery, the eyelid suture was taken out. The defects of the corneal epithelium were investigated by instilling 0.25% fluorescein sodium and photographed using a slit lamp (BQ900; Haag-Streit, Bern, Switzerland). The staining area was analyzed by using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and calculated.

Twenty-four mice were used for the corneal NAD$^+$ depletion model. FK866 (21 mM; ApexBio, Houston, TX, USA) dissolved in water containing 22.5% propanediol and 2.5% Tween 80 was subconjunctivally injected into the right eye of each mouse (5 lL/eye), and ofloxacin eye drops were subsequently applied to avoid infection for 2 consecutive days. The right eye of the normal mouse received water containing 22.5% propanediol and 2.5% Tween 80 as a vehicle control.

The eyeballs of denervated mice and corneal NAD$^+$ depletion mice were collected and dissected around the scleral-limbal region. The corneal epithelium was scratched surgically by an epithelial scraper and then used for RNA extraction and Western blotting.
Denervation Inhibits Epithelial NAD⁺ Biosynthesis

Corneal Sensitivity

Corneal sensation was detected using an esthesiometer (Cochet-Bonnet: Luneau Ophthalmologie, Chartres Cedex, France). The central area of cornea was touched by the maximal length (6 cm) of nylon filament and shortened by 5 mm each time until a blink response was observed. Corneal sensitivity was measured as the longest filament length that elicited positive response.

Quantification of Total NAD, NAD⁺, and NADH

After corneal denervation for 2 days, nine mouse eyeballs of denervated and control mice were collected and dissected around the scleral-limbal region. The corneal epithelium was scratched surgically by an epithelial scraper, and the corneal epithelial NAD⁺ and NADH were extracted using a NAD/NADH extraction buffer. The contents of total NAD, NAD⁺, and NADH were detected by a NAD/NADH quantification kit (Sigma-Aldrich Corp.). The total NAD, NAD⁺, and NADH levels were normalized to milligrams of wet epithelial weight.

Quantification of TRP, Kynurenine (KYN), and 3-Hydroxykynurenine (3-HK)

The cornea of each denervated and control mouse was homogenized in an extract buffer (water/methanol, 1:3 vol/vol) and in a mixed internal standard application liquid. After centrifugation, the supernatant was evaporated to dryness under vacuum. A sodium bicarbonate buffer (0.2 M, pH 11) and dansyl chloride in acetone (2 mg/mL) were then added to the dry residues for derivatization. The sample or standard substance was injected into ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometry and the data were analyzed by software (Thermo Xcalibur 2.2; Thermo Fisher Scientific, Waltham, MA, USA).

TUNEL Assay

Apoptosis was measured by TUNEL assay using a kit (In Situ Cell Death Detection Kit; Roche Diagnostics GmbH, Mannheim, Germany). Briefly, the corneal sections were fixed with 4% paraformaldehyde for 15 minutes and permeabilized in 0.1% Triton X-100 in a 0.1% sodium citrate solution for 2 minutes on ice. The sections were then incubated with TUNEL reaction solution at 37°C for 1 hour and observed though a microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan).

Cell Culture and Treatment

Human corneal epithelial cells (HCECs), provided by Choun-Ki Joo (The Catholic University of Korea, Seoul, Korea), were cultured as previously described. The HCECs were seeded in the six-well plate, allowed to adhere, and then treated with 10 nM FK866, 10 nM FK866 and 1.6 µM NMN, or 10 nM FK866 and 800 nM NAD⁺, in the serum-free medium for 24, 48, and 72 hours.

Cell Apoptosis Assay

The percentage of HCECs undergoing apoptosis was investigated by using a detection kit (apopNexin Annexin V-FITC Apoptosis Detection Kit; EMD Millipore, Lake Placid, NY, USA). The HCECs were trypsinized and incubated with annexin V and propidium iodide (PI) for 15 minutes and analyzed by flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) to determine the percentage of apoptotic cells in each sample.

Immunofluorescent Staining

The corneal whole-mount staining was performed as previously described. Briefly, the corneal flat mount was fixed in 4% paraformaldehyde for 1 hour and permeabilized in 0.2% Triton X-100 for 1 hour followed by 5% bovine serum albumin (Solarbio, Beijing, China) blocking for 1 hour at room temperature. Subsequently, it was incubated with Alexa Fluor 488 conjugated anti-βIII-tubulin antibody (EMD Millipore) for 12 hours at 4°C. For immunofluorescence staining, the sections were fixed in 4% paraformaldehyde for 15 minutes and permeabilized in 0.1% Triton X-100 for 15 minutes, followed by 5% bovine serum albumin blocking for 1 hour at room temperature. Sections were incubated with anti-NAMPT (Proteintech, Chicago, IL, USA) overnight at 4°C and then incubated with fluorescein-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour, followed by fluorescent stain (Hochest 33258; Solarbio) staining for 5 minutes. All staining was examined under an Eclipse TE2000-U microscope.

Real Time-Polymerase Chain Reaction

The total RNA extraction and cDNA synthesis of the corneal epithelium were performed using an amplification kit (Single Cell Sequence Specific Amplification Kit; Vazyme, Nanjing, China). Briefly, individual cells were harvested with 5 µL preamplification mix (RT-PreAmp Master Mix; Vazyme) and then frozen at −80°C for 2 minutes. The PCR procedures of reverse transcription and specific target amplification were performed as follows: 50°C for 60 minutes, 95°C for 3 minutes, 20 cycles at 95°C for 15 seconds, and 60°C for 15 minutes. The cDNA was diluted and amplified using the hot start reaction mix (FastStart Universal SYBR Green Master, Rox; Roche) and the primers as shown in the Supplementary Table.

Western Blotting Analysis

The total protein of the corneal epithelium was extracted and applied to 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes that had been pretreated with methanol (EMD Millipore). The PVDF membranes were incubated with anti-BAX (Abcam, Cambridge, MA, USA), anti-BCL-2 (Abcam), anti-GAPDH (Proteintech), anti-SIRT1 (Proteintech), anti-phospho-SIRT1 (Proteintech), anti-phospho-p38 (Proteintech), anti-phospho-S133 CREB (Abcam) or anti-CREB (Abcam) overnight at 4°C. Protein bands were incubated with methanol (EMD Millipore) and blocked with 10% nonfat milk for 1 hour at room temperature. Subsequently, the membranes were incubated with horseradish peroxidase (HRP) conjugated antibody (Proteintech), and the signal was detected by the enhanced chemiluminescence chemiluminescent system.

Statistical Analysis

All assays were performed in triplicate, and a 1-way ANOVA analysis was performed with statistical software (SPSS; IBM Corp., Armonk, NY, USA). A P value less than 0.05 was considered statistically significant; a P value less than 0.01 was considered extremely significant.

RESULTS

Corneal Denervation Causes Epithelial Detachment and Cell Apoptosis

To recapitulate corneal cell apoptosis by denervation, we modified the trigeminal axotomy previously described for the
The nerve bundles derived from the trigeminal ganglion were squeezed with forceps for 30 seconds. In the whole-mount corneal staining and fluorescein sodium staining, corneal nerve fibers degenerated significantly, while the epithelium detached spontaneously after 2 days of denervation treatment when compared with results of these tests on the sham control (Fig. 1B). Corneal sensitivity fell from 5.4 ± 0.1 mm in the control mice to 1.1 ± 0.1 mm in the denervated mice (Fig. 1C). In situ TUNEL staining identified the apoptosis of the epithelium, keratocytes, and endothelium (Fig. 1D), consistent with the previous description of corneal denervation. To confirm the apoptosis of corneal epithelial cells, the total RNA and protein of the corneal epithelium were collected for the analysis of qPCR and Western blotting at 2 days after the denervation treatment. The results showed that the mRNA transcript levels of proapoptotic gene Bax were upregulated by 5.1 fold, while the antiapoptotic gene Bcl-2 was downregulated by 7.7 fold when compared with the results for the control epithelium (Supplementary Fig. S1). Similar changes in the protein level were also observed by Western blotting (Fig. 1E). According to the protein expression levels, the ratio of BAX/BCL-2 was significantly higher for the denervated corneal epithelium (Fig. 1F). The results confirmed that corneal denervation caused epithelial detachment and cell apoptosis.

**Corneal Denervation Reduces Epithelial NAD$^+$ Levels**

Mitochondrial dysfunction caused by the depletion of intracellular NAD$^+$ has been reported to be involved in the initiation of apoptosis. To reveal the relationship of epithelial apoptosis and intracellular NAD$^+$ level after corneal denervation, the total NAD, NAD$^+$, and NADH of the corneal epithelium were measured and compared between the sham control mice and the denervated mice after 2 days of trigeminal axotomy. The results showed that after corneal denervation, the epithelial levels of the total NAD decreased from 12.5 to 7.4 pM/mg, NAD$^+$ decreased from 8.1 to 5.2 pM/mg, and NADH decreased from 4.4 to 2.2 pM/mg (Fig. 2).

**Corneal Denervation Impairs Epithelial NAD$^+$ Biosynthesis**

Intracellular NAD$^+$ is synthesized in mammalian cells by an eight-step de novo pathway derived from the TRP and salvage pathways from NA, NAM, or nicotinamide riboside (NR) (Fig. 3A). To demonstrate how corneal denervation reduces corneal NAD$^+$, three major components of the de novo pathway, including TRP, KYN, and 3-HK, were measured. The results showed that the levels of TRP, KYN, and 3-HK were significantly higher in the denervated cornea when compared with those in the control cornea (Supplementary Fig. S2A). In the corneal epithelium, the rate-limiting enzyme of the de novo pathway Qprt was lower by 1.5 fold (Supplementary Fig. S2B). Therefore, epithelial cell apoptosis induced by corneal denervation was accompanied by lower epithelial NAD$^+$ levels and impaired the de novo NAD$^+$ biosynthesis.

The salvage pathway plays a crucial pathway in the NAD$^+$ biosynthesis of mammalian cells. To illustrate the changes in the expression of key enzymes involved in the salvage pathway of NAD$^+$ biosynthesis, the total RNA of the corneal epithelium...
NAMPT Inhibition Disrupts the Homeostasis of the Corneal Epithelium

FK866, a selective inhibitor of NAMPT, has been used to deplete intracellular NAD⁺ in basic research and clinical trials.²⁴ To examine the role of NAMPT expression in the epithelial apoptosis of denervated mice, FK866 was injected subconjunctivally into normal mice. Similar to the treatment of denervated mice, FK866 treatment induced spontaneous epithelial detachment and resulted in epithelial defects (Fig. 4A). In situ TUNEL staining confirmed the apoptosis of the corneal epithelium, keratocytes, and endothelium in FK866-treated mice (Fig. 4B). The mRNA and protein levels of BAX were upregulated, while BCL-2 was downregulated in the mouse corneal epithelium after FK866 treatment, compared with the results of these tests for the control epithelium (Supplementary Fig. S3; Fig. 4C). The ratio of BAX/BCL-2 was essentially limited to the corneal epithelium, and it decreased in the denervated epithelium (Fig. 4C). Western blotting confirmed the lower NAMPT protein level after denervation (Fig. 3C). Therefore, we conclude that corneal denervation impairs the expression of NAMPT in the corneal epithelium.

NMN or NAD⁺ Improves Corneal Epithelial Defects by Denervation

In view of the apoptotic inhibition by NAD⁺ replenishments in cultured cells, we explored whether exogenous NMN or NAD⁺ could alleviate the spontaneous epithelial detachment and slow down the loss of the corneal nerve fibers induced by corneal denervation. As shown in Figure 6A, epithelial detachment was improved by the protective effects of NMN and NAD⁺. According to the analysis of epithelial defects, the treatment of replenishing NMN or NAD⁺ reduced the wound area in the denervated mice to only 9% at day 1 and 25% to 40% at day 2 of the respective values on these tests for the vehicle control (Fig. 6B). The corneal nerve fibers degeneration was slowed down by replenishing NMN or NAD⁺. The nerve fibers innervations were correlated with the health of epithelium (Fig. 6C). In addition, the epithelial apoptosis mainly occurred at the position of the epithelium detachment in the treated and untreated mice (Supplementary Fig. S5).
DISCUSSION

We used the corneal denervation experimental mouse model to clarify the role of epithelial innervations in maintaining its homeostatic functions. In the present study, the corneal denervation impaired the epithelial NAD$^+$ contents by reducing the expression of NAMPT, the rate-limiting enzyme of NAD$^+$ biosynthesis. Moreover, we confirmed that the NAD$^+$ depletion by NAMPT inhibition resulted in the deactivation of SIRT1, pAKT, and pCREB and caused the apoptosis of corneal epithelial cells. In addition, the replenishment of NMN or NAD$^+$ attenuated epithelial detachment in the corneal denervated mice and attenuated the induction of apoptosis in the NAD$^+$-depleted cells. The results suggest that corneal innervations maintained the epithelial homeostasis by regulating the expression of NAMPT and by regulating the level of intracellular NAD$^+$, whereas the denervation caused epithelial apoptosis through the impaired NAD$^+$ biosynthesis (Fig. 7).

Peripheral nerve injury induces Wallerian degeneration of severed axons distal to the injury site. Wallerian degeneration comprises two phases: the latent period and the active period. In cultured dorsal root ganglion neurons, the latent period precedes morphologic changes and usually begins within approximately 4 to 6 hours, accompanied by NMNAT2 depletion and transient phosphorylation of MKK4 at S257/T261. In this period, NAD$^+$ declines from approximately 5 to 6 hours, which results in the further decline of ATP levels and neurofilament proteolysis, leading to an active and irreversible phase of axon degeneration. In neurodegenerative diseases, the reduction of the NAD$^+$ content disturbs the mitochondrial energy metabolism of the axon and causes neuronal cell death and axonal degeneration, such as in Alzheimer's and Parkinson's diseases. The regulation of the metabolic NAD$^+$, which plays a vital role in energy metabolism and participating in metabolic processes, tightly controls the metabolism, health, and life span. The depletion of intracellular NAD$^+$ impairs the capacity of the energy metabolism and leads to cell apoptosis. Among the NAD$^+$-dependent protein deacetylase of the SIRTs family, SIRT1 has been found to be a key regulator of cells survival by enhancing the phosphorylation of AKT at Ser473, which plays a central role in promoting survival and preventing apoptosis of a wide range of cell types. Moreover, the AKT-dependent pathway promotes cell survival by stimulating cellular gene expression through the transcription factor CREB phosphorylation at Ser133. CREB leads to an increase in antiapoptotic protein BCL-2 expression by a CRE located at 1640 to 1529, which plays a crucial role in regulating cell death via stabilization of the mitochondria membrane potential. In addition, the AKT pathway inhibits a conformational change of the proapoptotic protein BAX that subsequently translocates to mitochondria, thus inhibiting apoptosis by preventing alterations in the mitochondrial membrane potential. Meanwhile, NAMPT has been found to protect cells against apoptosis by activating the AKT pathway, increasing the expression of BCL-2, and decreasing the
expression of BAX. Therefore, apoptosis may be caused by the decreasing of NAD, SIRT1, p-AKT, p-CREB; further increased the expression of BCL-2; and decreased the expression of BAX that maintained the epithelial homeostasis (left panel). Corneal denervation impaired the expression of NAMPT and NAD, which caused the apoptosis of corneal epithelial cells. The replenishment of NMN or NAD restored the activation levels of SIRT1, AKT, and CREB and reversed the apoptosis of corneal epithelium (right panel).

**FIGURE 6.** Exogenous NMN or NAD attenuated epithelial defect by corneal denervation. (A) Epithelial defects of corneal denervated mice with or without exogenous NMN or NAD treatment were detected by fluorescein sodium staining at day 1 and 2. (B) Quantification of epithelial wound area. (C) Corneal nerve of corneal denervated mice with or without exogenous NMN or NAD treatment was detected with whole-mount βIII-tubulin staining at day 1 and 2. Data are representative of means ± SEM. **P < 0.01.

**FIGURE 7.** Schematic model of corneal epithelial homeostasis maintained by innervations. Corneal innervations regulated the expression of NAMPT in the corneal epithelium, which was important to NAD biosynthesis. The intracellular NAD regulated the activation of SIRT1, pAKT, pCREB; further increased the expression of BCL-2; and decreased the expression of BAX that maintained the epithelial homeostasis (left panel). Corneal denervation impaired the expression of NAMPT and NAD, which caused the apoptosis of corneal epithelial cells. The replenishment of NMN or NAD restored the activation levels of SIRT1, AKT, and CREB and reversed the apoptosis of corneal epithelium (right panel).

**Acknowledgments**

Supported by grants from the National Natural Science Foundation of China (81530027, 81770904, 81670829, and 81670828). Qingjun Zhou and Weiyun Shi are partially supported by the Taishan Scholar Program (20150215 and 20161059) and the Innovation Project of Shandong Academy of Medical Sciences.

**Disclosure:** Y. Li, None; X. Ma, None; J. Li, None; L. Yang, None; X. Zhao, None; X. Qi, None; X. Zhang, None; Q. Zhou, None; W. Shi, None
References


