Original Article

Analysis of the Activation of the Nrf2-ARE Pathway Following Optic Nerve Injury in Mice

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Abstract

Purpose: The Nrf2-ARE pathway plays a cytoprotective role in many tissues, but its protective function in the optic nerve is unclear. The purpose of the study is to investigate the changes in activation of the Nrf2-ARE pathway following optic nerve injury (ONI) in mice.

Methods: Using ONI mice models, the expression levels of Nrf2 in optic nerves were determined by real-time PCR at various time points.

Results: The expression of Nrf2mRNA was significantly up-regulated at 1 d after ONI, peaking at 30 min after ONI.

Conclusion: The Nrf2-ARE pathway was activated after ONI, providing evidence for the study of the protection and underlying mechanism of Nrf2-ARE pathway on optic nerves. (***Eye Science 2012*; *27*: *161–164***)

Keywords: optic nerve; injury; protection; Nrf2

Optic nerve injury (ONI) can be caused by a variety of factors including trauma, ocular hypertension, ischemia, and tumor compression, which lead to irreversible visual function damages and even permanent blindness (70%). However, no effective treatment has yet been established for optic nerve injuries.

A series of key steps is involved in promoting the healing of optic nerve injuries, including activation of underlying protective mechanisms, easing of secondary damage, enhancing the survival of ganglion cells, and controlling the expression of apoptosis genes. Nuclear factor E2-related factor 2 (Nrf2) is a newly discovered transcription factor that couples with Kelch-like ECH-associated protein1 (Keap1) and is not activated. Confronted by various stimulations, Nrf2-Keap1 degrades in the cytoplasm and translocates into the nucleus. In the nucleus, Nrf2 binds to the Antioxidant Response Element (ARE) in the upstream promoter region of many anti-oxidative genes, initiates their transcription, and increases the defense immunity against external stimuli[7–9]. The Nrf2-ARE pathway is a regulatory mechanism in cells that provides simultaneous regulation of multiple illnesses and plays a protective role in many cases. In this study, the Nrf2-ARE pathway is assumed to be capable of alleviating secondary injuries and generating a self-protective effect in nerve cells. However, relevant studies have not been performed yet in China. Therefore, the activation of the Nrf2-ARE pathway was investigated by Real-time PCR after ONI. The findings reported here provide clinical evidence for the potential value of analyzing the protective role of the Nrf2-ARE pathway in ONI.

Materials and methods

Establishment of ONI mice models

ICR mice were bred by the Experimental Animal Center of Nanjing General Hospital of the Nanjing Military Command. The ONI mice models were established by damaging the optic nerve with forceps. ICR mice were randomly divided into operation and sham-operation groups (n=10 for each group). After sterilization and anesthesia, the bulbar conjunctival fornix was cut open, the optic nerves in both eyes were separated and exposed, the optic nerve sheath was incised, the optic nerve was vertically clipped by a 40g optic nerve clamp (OX60, Germany) for 30 s about 2 mm posterior to the eyeball in the right eye, and the untreated left eye was used as a control.

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The bulbar conjunctival incision was sutured and aureomycin ointment was applied. All optic nerve injuries were made at 2 mm from the retrobulbar site to avoid damaging the retinal central blood system. After surgery, the models with Marcus–Gun pupil and full eyelid closure and with no obvious exophthalmos or fundus hemorrhage were included in further experiments. The mouse models were sacrificed at various time points after ONI (30 min, 1 h, 6 h, 12 h, and 1 d), the left ventricular was cut open for bleeding, eyes were enucleated, and then the optic nerves were separated for subsequent analysis. Twenty mice were used for each time point, randomly divided into two groups, and the experiment was repeated three times.

Real-time PCR assay

For extraction of total RNA, the optic nerves of the mice in both groups were collected at various time points, washed in DEPC, and extracted in Trizol reagent. Reverse transcription was conducted using a SuperScript III first strand kit (Invitrogen, Carlsbad, CA, USA). A Light Cycler 480 SybrGreen I Master kit (Roche) and the 7900HT Fast Real–Time PCR System were utilized for Real–time PCR assays. The Nrf2 primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Forward: 5'-TCAGCGACGGAAAGAGTATGA-3'
Reverse: 5'-CCACTGGTTTCTGACTGGATGT-3'
GAPDH was used as an internal control.
Forward: 5'-GAAATCCCATCACCATCTTC-3'
Reverse: 5'-GGACTCCACGACGTACTCA-3'

PCR reaction system was a FastStart Universal SYRB Green Master, with forward primer (15 μmol), reverse primer (15 μmol), cDNA, and DEPC. The PCR reaction conditions were denaturation at 95℃ for 10 min, 95℃ for 15 s, and 58℃ for 45 s, for 45 cycles. The Ct value was calculated according to the PCR reaction curve and relative quantitative data were analyzed by the 2-△△Ct method.

Statistical analysis

The data were analyzed using SPSS17.0 statistical software. All data were expressed as mean ±s.d. Multi-group comparisons were analyzed by ANOVA. $P<0.05$ was considered as statistically significant.

Results

Activation of the Nrf2-ARE pathway after ONI in mouse models

Under normal conditions, low expression of Nrf2 was observed in mice, while it was activated after ONI in a short time, peaked at 30 min after ONI, and subsequently presented with a decreasing trend.

Expression of Nrf2mRNA peaked at 30 min after ONI

The expression level of Nrf2mRNA was significantly upregulated at 1 d after ONI ($P<0.05$), and peaked at 30 min after ONI ($P<0.01$).

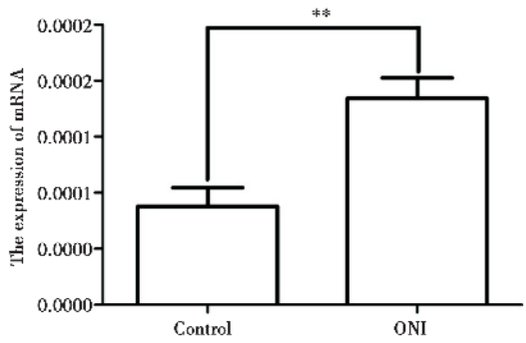

Figure 1 Changes in the expression of Nrf2mRNA after ONI in mouse models. Real-time PCR assays revealed higher expression of Nrf2mRNA at 30 min post ONI compared to the control group ($n=10$, $^{**}P<0.01$ versus control group).

Discussion

The optic nerve is composed of retinal ganglion cell (RGC) axons. ONI and axonal injury directly disrupt the integrity of the RGC membrane and create nutritional factor deficiencies. Rapid biochemical responses of RGCs are activated by stress responses, which lead to fast degeneration and death of these cells. Injured RGCs release cytotoxins, oxygen free radicals, and inflammatory factors, and cause Ca^{2+} overload, initiating the programming of RGC apoptosis and causing secondary injuries, predominantly apoptosis[1,2]. Therefore, anti-oxidative stress, inflammatory response regulation, resistance to cell apoptosis, and alleviation of Ca^{2+} overload play key roles in relieving secondary ONI, enhancing the survival of ganglion cells, and promoting the repair of ONI.

Even the most commonly used hormone therapy or the most advanced gene therapy target only one or

several mechanisms, but are unable to solve the complicated network of the various mechanisms involved in ONI. Much attention has been paid to the mechanism of secondary injuries rather than the underlying physical aspects that cause adverse events and negatively affect recovery. The Nrf2-ARE pathway is a cellular regulation mechanism and its activation can occur under normal conditions, leading to expression of a variety of factors including phase II detoxifying enzymes, antioxidant enzymes, anti-inflammation proteins, growth factors, and DNA repair enzymes. It initiates a series of defense mechanisms against secondary injuries such as initiating anti-oxidative stress, regulating the inflammatory response, resisting cell apoptosis, and easing Ca^{2+} overload, etc[3–6]. The Nrf2-ARE pathway has the following characteristics[7,8]: first, the pathway is an internal regulation mechanism and promotes the expression of different proteins at various levels depending on the cell type and the external stimulus. Second, the Nrf2-ARE pathway, although only one target, enables organs and tissues to express a variety of protective proteins that play a coordinated role in multiple events, such as lipid peroxidation, calcium homeostasis, inflammatory responses, cell apoptosis, and metabolism.

Previous studies systematically analyzed the protective effect of the Nrf2-ARE pathway after brain and spinal cord nerve injuries and found that this pathway was activated after brain injuries and it exerted a protective effect on injured secondary nerve cells by anti-inflammation and alleviating oxidation stress[9–11]. However, few studies have reported the effect of Nrf2-ARE pathway on ONI models. One previous study suggested that both in vivo and in vitro genipin induced the production of HO-1 to protect RGCs from oxidative stress via the Nrf2-ARE pathway[12].Another investigation confirmed that HNE induced the synthesis of Nrf2 and promoted the secretion of GSH and antioxidant enzymes to alleviate oxidative stress-induced ONI[13].

Some scholars compared Nrf2-/-ischemia mouse models and perfusion retinal injury models and noted that the Nrf2-ARE pathway played a vital role in preventing retinal injuries and therefore represented a novel therapeutic target[14].The present study showed that the Nrf2-ARE pathway was activated after ONI and the expression level subsequently peaked at 30 min. These findings provide experimental evidence to support the analysis of its protective function and underlying mechanism and the exploration of novel medicines for ONI that target the Nrf2-ARE pathway.

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Name Change Notification

Effective from 8 December 2012, Tianjin Medical University Eye Centre will change its name to "Tianjin Medical University Eye Hospital" (TMUEH).

This name change is being instituted to reflect the Hospital's recently enhanced roles in providing a full range of ophthalmic care from comprehensive to tertiary levels for the entire spectrum of ophthalmic diseases with emphasis on quality and excellency.

With the accomplishment of our 3rd phase of expansion in May 2012,we established a multidisciplinary framework for our clinical services, research institution and medical education system, which is referred to as "8·1·1·4".

"8" refers to eight clinical subspecialties: Cataract, Glaucoma, Cornea & Refractive, Vitreo–Retinal, Ocular Fundus and Neuro–ophthalmology, Ocular Trauma and Ocular Plastic and Orbital Disease, Uveitis and Immuno–ophthalmology, and Traditional Chinese Ophthalmology.

"1·1" refers to the Optometry Department and Tianjin Medical University Eye Institute.

"4" refers to our four centers: the Diabetic Eye Disease Prevention and Control Center, Excimer Laser Treatment Center, Strabismus and Pediatric Eye Disease Treatment Center and Low Vision Rehabilitation Center.