Minocycline inhibits the production of the precursor form of nerve growth factor by retinal microglial cells

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Abstract
A rat model of acute ocular hypertension was established by enhancing the perfusion of balanced salt solution in the anterior chamber of the right eye. Minocycline (90 mg/kg) was administered intraperitoneally into rats immediately after the operation for 3 consecutive days. Immunofluorescence, western blot assay and PCR detection revealed that the expression of the precursor form of nerve growth factor, nerve growth factor and the p75 neurotrophin receptor, and the mRNA expression of nerve growth factor and the p75 neurotrophin receptor, increased after acute ocular hypertension. The number of double-labeled CD11B- and precursor form of nerve growth factor-positive cells, glial fibrillary acidic protein- and p75 neurotrophin receptor-positive cells, glial fibrillary acidic protein- and caspase-3-positive cells in the retina markedly increased after acute ocular hypertension. The above-described expression decreased after minocycline treatment. These results suggested that minocycline inhibited the increased expression of the precursor form of nerve growth factor in microglia, the p75 neurotrophin receptor in astroglia, and protected cells from apoptosis.

Key Words
neural regeneration; biological factor; precursor form of nerve growth factor; p75 neurotrophin receptor; minocycline; apoptosis; nerve growth factor; acute ocular hypertension; retina; photographs-containing paper; neuroregeneration

Research Highlights
(1) Retinal microglia are an important source of the retinal precursor form of nerve growth factor after acute ocular hypertension.
(2) Retinal astroglial apoptosis after acute ocular hypertension was associated with the increased expression of the precursor form of nerve growth factor and the p75 neurotrophin receptor.
(3) The neuroprotective effect of minocycline was due to the inhibiting effect of the precursor form of nerve growth factor and the p75 neurotrophin receptor.

INTRODUCTION
Glaucomatous neurodegeneration is a progressive pathological affair characterized by retinal ganglion cell death resulting in functional visual deterioration[1-3]. Glial cells are structurally and functionally linked to neuronal tissues. In glaucoma, pathologic mechanisms may include glial activation[4].

At the early stages of a pressure rise, there is both astroglial and axonal injury in neural tissue[4-5]. The amount of astroglia excised from the glaucoma-affected retina is reduced[6]. In addition, microglia have been
detected on the retinal surface as early as 72 hours after induction in a rat model of glaucoma, and activation of intraretinal microglia coincided with the degeneration of retinal ganglion cells in glaucoma[7]. Microglial activity remained elevated, even after intraocular pressure returned to basal levels. The degree of activation in the optic nerve correlated with axonal damage[8]. Therefore, both changes in astroglial and microglial cells may be involved in glaucomatous neurodegeneration. In many neurodegenerative diseases, astroglia are associated with recovery, whereas microglia are related to occurrence and development[9-10]. However, the role of the two cells in glaucoma and the exact connections between them remain unknown.

The precursor form of nerve growth factor, the predominant nerve growth factor moiety, found in the human brain[11-12], can be induced by different injuries to the nervous system[13-15]. Recent studies showed that cell-surface interactions between precursor form of nerve growth factor and its high affinity receptor, p75 neurotrophin receptor, facilitated the pro-apoptotic signaling cascade[16-19]. Microglial production of precursor form of nerve growth factor increased when the nervous system was injured[20]. Nevertheless, whether retinal microglial production of precursor form of nerve growth factor after acute ocular hypertension is associated with the retrogressive changes of astroglia has not been investigated.

Minocycline, a derivative of tetracycline, mediates neuroprotection in many neurodegenerative diseases[21-22]. It reduced microglial activation and improved retinal ganglion cell axonal transport and integrity in a glaucomatous model[23]. However, the mechanism has not been identified. Because minocycline significantly reduced precursor form of nerve growth factor production in microglia and inhibited p75 neurotrophin receptor expression in macroglia after spinal cord injury[24], this study examined whether minocycline mitigated the damage of retinal astroglia by inhibiting precursor form of nerve growth factor production in microglia after acute ocular hypertension.

RESULTS

Quantitative analysis of experimental animals

One hundred and sixty rats were equally and randomly assigned to four groups: sham-operated group, model group, minocycline group and PBS group. The latter three groups were perfused with balanced salt solution in the anterior chamber of the right eye with a hanging saline bottle at a height of 146 cm to establish an intraocular pressure model. The minocycline group and PBS group were administered intraperitoneally with minocycline and PBS, respectively. Among the total number of rats, four died during the anesthesia and five developed cataracts after operation. All nine of these rats were rejected from the final analysis and the study was supplemented with additional animals. In total, 160 rats were included in the final analysis. Ten rats from each group were analyzed at 1, 3, 5 and 7 days following model induction.

Minocycline inhibited precursor form of nerve growth factor expression in rat retinal microglia after acute ocular hypertension

Western blot assay showed that both nerve growth factor and precursor form of nerve growth factor in the retina were induced markedly after acute ocular hypertension (Figure 1).

The expression of precursor form of nerve growth factor increased from 1 day after acute ocular hypertension and peaked at 3 days, and then decreased gradually from 5 days. There was no expression of nerve growth factor in the sham-operated group at all time points or in the model group at 1 and 3 days, and until 5 days. Reverse transcription-PCR analysis revealed that nerve growth factor mRNA expression in the retina increased and peaked at 3 days (Figure 2).
At 3 days after acute ocular hypertension, double labeling for precursor form of nerve growth factor and CD11B, a marker of microglia, was observed in the retinal ganglion cell layer, outer plexiform layer and outer nuclear layer, especially in the outer plexiform layer, but was not observed in sham-operated rats (Figure 3).

Treatment with minocycline significantly inhibited retinal precursor form of nerve growth factor protein expression at 3 days after acute ocular hypertension (Figures 1, 2), as well as double labeling of precursor form of nerve growth factor- and CD11B-positive cells in the ganglion cell layer, outer plexiform layer and outer nuclear layer, especially in the outer plexiform layer (Figure 3).

**Minocycline inhibited p75 neurotrophin receptor expression in retinal astroglial cells after acute ocular hypertension**

Both p75 neurotrophin receptor protein and mRNA expression increased after acute ocular hypertension (Figures 1, 2). Western blot assay and reverse transcription-PCR analysis showed that p75 neurotrophin receptor protein and mRNA expression increased as early as 1 day after acute ocular hypertension, peaked at 3 days, then began to decrease gradually from 5 days. Immunohistochemistry revealed double labeling of p75 neurotrophin receptor and glial fibrillary acidic protein, a marker of astroglia. Positive staining was observed in the retinal ganglion cell layer at 3 days after acute ocular hypertension, but not in the control (Figure 4).

Western blot assay and reverse transcription-PCR analysis showed that the production of p75 neurotrophin receptor induced by acute ocular hypertension was reduced considerably after minocycline treatment at 3
days (Figures 1, 2), and that double labeling in the ganglion cell layer also decreased (Figure 4).

**Minocycline reduced apoptosis of retinal astroglial cells after acute ocular hypertension**

Double labeling immunohistochemistry revealed that some glial fibrillary acidic protein-positive cells in the rat retinal ganglion cell layer were also positive for caspase-3 at 3 days after acute ocular hypertension, whereas these double-labeled cells were not obvious in sham-operated rats. After treatment with minocycline, glial fibrillary acidic protein- and caspase-3-positive cells were not observed at 3 days in the acute ocular hypertension-induced rat retina (Figure 5).

![Double labeling of glial fibrillary acidic protein (GFAP) and caspase-3 in astroglial cells (fluorescent microscope). GFAP-positive cells (green, arrow, DyLight™488 staining). Caspase-3 positive cells (red, arrow, DyLight™594 staining). GFAP- and caspase-3-positive cells were observed after acute ocular hypertension. Minocycline inhibited co-labeling of GFAP and caspase-3 after acute ocular hypertension. Cells stained blue with 4',6-diamidino-2-phenylindole. GCL: Ganglion cell layer. Scale bars: 25 μm.](image)

The relative levels of precursor form of nerve growth factor receptors determine whether the precursor exhibits neurotrophic or apoptotic activity. Precursor form of nerve growth factor induces apoptosis when high affinity receptor p75 neurotrophin receptor levels are high\[^{27-28}\]. Previous studies illustrated that protein expression of p75 neurotrophin receptor in the ischemic retina of rats increased, as determined by western blot assay and immunohistochemistry\[^{18, 29}\]. This study demonstrated that not only p75 neurotrophin receptor protein but also mRNA expression increased in the retina after acute ocular hypertension. In addition, p75 neurotrophin receptor and glial fibrillary acidic protein were coexpressed in the retinal ganglion cell layer, which indicated that p75 neurotrophin receptor expression was elevated in retinal astroglial cells after acute ocular hypertension. Consequently, precursor form of nerve growth factor could mediate apoptosis.

Precursor form of nerve growth factor in neurons and their adjacent glial cells reduced its biological role by binding to excreted neurocytes\[^{32, 33}\]. However, the cell types producing precursor form of nerve growth factor during acute ocular hypertension have not been identified. Microglia can produce precursor form of nerve growth factor when stimulated by inflammation and trauma\[^{26}\]. Moreover, microglia are activated following glaucoma\[^{30}\]. Therefore, we examined whether microglia express precursor form of nerve growth factor after acute ocular hypertension. In the present study, large amounts of precursor form of nerve growth factor were expressed in the retinal ganglion cell layer, outer plexiform layer and outer nuclear layer at 3 days after acute ocular hypertension, especially in the outer plexiform layer. Additionally, CD11B, a marker of microglia, was also coexpressed. Thus, our data indicate that activated microglia appear to be an important source of precursor form of nerve growth factor.

**DISCUSSION**

In glaucoma, retinal ganglion cell death may be due to astroglial impairment\[^{4, 6, 25}\]. The mechanism underlying astroglial impairment has not been identified. Precursor form of nerve growth factor as the precursor of nerve growth factor is secreted by many tissues and is the predominant form of nerve growth factor in the central nervous system\[^{26}\]. The role of this precursor is controversial, and both neurotrophic and apoptotic activities have been reported for recombinant precursor form of nerve growth factors. Therefore, it will be beneficial to find out the mechanism of the glial change in glaucoma by demonstrating the level of precursor form of nerve growth factor. Here, our data showed that acute ocular hypertension induced retinal precursor form of nerve growth factor production, which peaked at 3 days after acute ocular hypertension. Gial fibrillary acidic protein- and caspase-3-positive cells were observed in the ganglion cell layer at 3 days, whereas precursor form of nerve growth factor expression remained low and double-labeled positive cells were seen in the control. These results indicate that acute ocular hypertension induces retinal precursor form of nerve growth factor expression, which is associated with retinal astroglial apoptosis.
form of nerve growth factor, which contributes to the death of astroglia after acute ocular hypertension.

Glaucomatous astroglia impairment was characterized by morphological changes, and reduction and changes in density of glial fibrillary acidic protein staining[4, 7]. We demonstrated that retinal astroglia expressed caspase-3 at 3 days after acute ocular hypertension, which is coincident with the peak of precursor form of nerve growth factor and p75 neurotrophin receptor expression. Thus, our data suggest that acute ocular hypertension induced retinal microglia to express precursor form of nerve growth factor and astroglia to express its high affinity receptor p75 neurotrophin receptor, which bind to each other and induce apoptosis of astroglial cells after acute ocular hypertension.

The neuroprotective effect of minocycline in neurodegenerative diseases has been confirmed before[21, 33]. Minocycline inhibited apoptosis of retinal neurocytes cultured at high pressure in vitro[34]. The neuroprotective mechanism of minocycline has been reported to involve the inhibition of microglia function[35]. In this study, minocycline was administered intraperitoneally into acute ocular hypertensive rats immediately after operation. Subsequently, precursor form of nerve growth factor protein and nerve growth factor mRNA expression in the rat retina decreased significantly. Glial fibrillary acidic protein- and caspase-3-positive expression also reduced. These results revealed that minocycline reduced retinal astroglial apoptosis at least partly by inhibiting precursor form of nerve growth factor production by microglia after acute ocular hypertension. Overall, there was a decrease in retinal p75 neurotrophin receptor protein and mRNA expression, as well as double labeling of p75 neurotrophin receptor- and glial fibrillary acidic protein- positive cells, which implied that minocycline-mediated apoptosis had an intimate relationship with p75 neurotrophin receptor expression in astroglia.

In summary, the mechanism of astroglial apoptosis after acute ocular hypertension and the underlying neuroprotective mechanism of minocycline were explained. The increase of p75 neurotrophin receptor in astroglia is crucial for its induction of precursor form of nerve growth factor and exhibit apoptotic activity in acute ocular hypertension. The mechanism by which minocycline reduces apoptosis of astroglia is associated with the inhibition of precursor form of nerve growth factor from microglia and p75 neurotrophin receptor in astroglia. Minocycline may be a beneficial drug in humans to inhibit astroglial apoptosis in glaucoma.

**MATERIALS AND METHODS**

**Design**
A randomized, controlled, animal experiment.

**Time and setting**
The experiment was performed at the Kunming Institute of Zoology, Chinese Academy of Sciences in China from August 2010 to February 2011.

**Materials**
Healthy, adult, male, clean, Sprague-Dawley rats (n = 160), weighing 250–300 g and aged 3–4 months, were supplied by Kunming Medical College, China, license No. SYXX (Dian) 2005-0003. They were raised in a clean animal room with free illumination, at 20–23°C and humidity at 40–80%. Principles of laboratory animal care were in accordance with the guidelines expounded by the European Community Council Directive (86/609/EEC of 24 November 1986) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Methods**

**Modeling of intraocular pressure**
All animals were anesthetized with chloral hydrate (500 mg/kg) and 0.04% (v/v) benzoyl (Santen, Suzhou, China). The pupil was enlarged following treatment with mydrin (Santen, Suzhou, China). In the model group, the intraocular pressure was raised to 14.67 kPa for 60 minutes by enhancing the perfusion of balanced salt solution (Nanjing, Kunming, China) with a 27-gauge needle placed in the anterior chamber of the right eye. The intraocular pressure was achieved by hanging a saline bottle at the height of 146 cm. All procedures were performed under sterile conditions and did not injure any intraocular structures. For the sham-operated controls, the eyes were perfused only, with a bottle hanging at the same height as the operated eye[36].

**Minocycline intervention**
In the minocycline group, minocycline (90 mg/kg; Sigma, Santa Clara, CA, USA) was administered intraperitoneally in sterile PBS (20 g/L) into rats immediately after operation for 3 consecutive days. In the PBS group, 0.1 M PBS (4.5 mL/kg) was administered into the other animal models in the same way.
Sample collection
The rats were decapitated following anesthesia with chloral hydrate at 1, 3, 5 and 7 days.

Immunohistochemical detection of p75 neurotrophin receptor, precursor form of nerve growth factor, glial fibrillary acidic protein and CD11B in the rat retina
The eyeballs were excised out and fixed with 4% (w/v) paraformaldehyde (pH 7.4) for 1 hour on ice and then for another half an hour after removal of the cornea, lens and vitreous body. After that, the calyculi ophthalmicuses were placed in 10% (w/v) sucrose julep (pH 7.4) at 4°C to dewater, and then in 20% (w/v) and finally 30% (w/v). Tissues were embedded and cut into transverse frozen slices of 6–10 μm. The frozen slices were incubated with 2% (w/v) normal bovine serum albumin and 0.4% (w/v) Triton X-100 for 1 hour then with rabbit anti-p75 neurotrophin receptor (1:100; Millipore, Billerica, MA, USA) or precursor form of nerve growth factor (1:200; Millipore) polyclonal antibodies, or mouse anti-rat glial fibrillary acidic protein (1:200; Millipore) or CD11B (1:100; Chemicon, Billerica, MA, USA) monoclonal antibodies at 4°C overnight, followed with DyLight™ 594-conjugated goat anti-rabbit IgG (1:200; ZSBG-Bio, Beijing, China) or DyLight™ 488-conjugated goat anti-mouse IgG (1:200; ZSBG-Bio, Beijing, China) for 1 hour at room temperature. For double labeling, two kinds of primary antibodies or secondary antibodies were incubated at the same time. Nuclei were labeled with 4′,6-diamidino-2-phenylindole in fluorescent mounting medium (ZSBG-Bio, Beijing, China). In controls, the slices were incubated without primary antibodies. A DX60 fluorescent microscope (Olympus, Jiangsu, China) was used.

Western blot detection of p75 neurotrophin receptor, precursor form of nerve growth factor and nerve growth factor in the rat retina
Fresh retinal tissues were homogenized in radioimmune precipitation assay lysis buffer for 30 minutes on ice, then centrifuged at 25 000 × g for 30 minutes at 4°C. Equal amounts of protein were determined using bicinchoninic acid assay37 (Pierce, Pittsburgh, PA, USA). Protein samples (40 μg) were separated on 12% SDS-polyacrylamide gels after being denatured at 95°C for 5 minutes, and then transferred to polyvinylidene difluoride membranes (Roche, Shanghai, China). The membranes were incubated with 5% (w/v) nonfat skim milk in Tris-buffered saline Tween-20 for an hour at room temperature, and then with the primary antibodies rabbit anti-p75 neurotrophin receptor (1:4 000), precursor form of nerve growth factor (1:4 000) polyclonal antibodies, or mouse anti-rat glial fibrillary acidic protein monoclonal antibody (1:2 000), and finally with horseradish peroxidase-labeled goat anti-rabbit monoclonal antibody (1:10 000, KPL, Gaithersburg, MD, USA). Immunoblots were detected by chemiluminescence (SuperSignal, Pierce). The experiment was repeated in triplicate. Rabbit against rat β-actin monoclonal antibody (1:8 000; Millipore) was used as the internal control. The UVP BioDoc-IT™ gel-imaging instrument (UVP Inc., San Gabriel, CA, USA) was used.

Reverse transcription-PCR detection of p75 neurotrophin receptor and nerve growth factor in the rat retina
Total RNA was isolated from the retina using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized according to the Platinum Taq DNA polymerase instructions (Invitrogen). Samples were denatured at 94°C for 5 minutes, followed by amplification for 30–35 cycles of 95°C for 30 seconds, 55–60°C for 40 seconds, 72°C for 30 seconds to 1 minute. After that, PCR products were subjected to electrophoresis on a 1.5% agarose gel and visualized using ethidium bromide. The primers were designed using the Oligo6.53 software (SinoGenoMax, Beijing, China).

The primer sequences are as follows:

| Primer       | Sequence                        | Products size (bp) |
|-------------|---------------------------------|--------------------|
| Nerve growth factor | Sense: 5'-GGG AGT AGA AAA GGG CTG AGC-3' | 293 |
|             | Antisense: 5'-TAT CCC ATG GGC AGT AAA GGA CA-3' | |
| p75 neurotrophin | Sense: 5'-AGC CAA CCA GAC CGT GTG TG-3' | 662 |
| receptor     | Antisense: 5'-TTG CAG CTC TTG CAC CTC TT-3' | |
| β-actin      | Sense: 5'-AAG ATC GTG CCC GAT GAA CGA GA-3' | 620 |
|             | Antisense: 5'-TCT TCT GGG TCC AGG AGC CGT G-3' | |

Negative controls consisted of PCR reactions lacking primers or reverse transcriptase. β-actin was used as the internal control. The experiment was repeated in triplicate. The UVP BioDoc-IT™ gel-imaging instrument (UVP Company) was used.

Apoptosis detection of astroglial cells in the rat retina
The expression of caspase-3 was used to test apoptosis38. Permeated and blocked frozen slices were incubated with primary mouse anti-rat glial fibrillary acidic protein monoclonal antibodies (1:200) and rabbit
anti-caspase-3 polyclonal antibodies (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at the same time at 4°C overnight, then incubated with DyLight™
594-conjugated goat anti-rabbit IgG (1:200) and
DyLight™ 488-conjugated goat anti-mouse IgG (1:200)
at room temperature for an hour. After mounting with
DAPI mounting medium, a DX60 fluorescent microscope
(Olympus) was used.

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guidance and manuscript editing. All authors have read and
agree to the manuscript as written.

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