·Basic Research·

Effects of brain-derived neurotrophic factor on the expression of caspase -2 and caspase -3 and cell apoptosis in retinal ischemia/reperfusion injury

Ying-Bin Xie, Ying-Jun Niu, Chun-Yan Yuan, Ying Yang, Wei-Yan Zhou, Xiu-Ting Yu

Department of Ophthalmology, Affiliated Hospital of Qingdao University Medical College, Qingdao 266003,Shandong Province, China

Correspondence to: Ying-Jun Niu. Department of Ophthalmology, Affiliated Hospital of Qingdao University Medical College, Qingdao 266003, Shandong Province, China. niuyingjun@yahoo.com.cn

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Abstract

• AIM: To explore the relationship between the expression of caspase-2 and caspase-3 and the apoptosis in retinal ischemia/reperfusion (I/R) injury of rats, as well as the therapeutic effects of brain derived neurotrophic factor (BDNF)on the ischemic and reperfused retina.

• METHODS: This experiment was conducted at the laboratory of Affiliated Hospital of Qingdao University Medical College from February 2007 to July 2007. The models of retinal ischemia/reperfusion injury were made by transiently elevating intraocular pressure. A total of 28 rats were divided into normal and operative groups. Operative group was divided into six subgroups. In each subgroup there were four rats. The left eyes of rats were used for I/R and the right eyes were used for intravitreal injection of brain-derived neurotrophic factor (BDNF) as treatment group. After reperfusion we divided our subgroups according to the reperfusion time as 1, 6, 12, 24, 48, 72 hours. The retinal ganglion cell number was counted by using optic microscope (BX-51,Olympus). Apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) method, and the expression of caspase-2, caspase-3 was studied by enzyme linked immunosorbent assay (ELISA) and strept avidin-biotin complex (SABC) immunohistochemistry.

• RESULTS: No positive apoptotic cells were observed in the normal rats' retinae, but there were a significant number of positive apoptosis cells in 6-24 hours after transient ischemia followed by a decrease at 48 hours. The number of apoptotic cells reached a maximum at 24 hours after ischemia .The expression of caspase-2 gradually increased as early as at 6 hours, reached a peak at 24 hours, then decreased between 48 and 72 hours. Similarly, caspase-3 has the same rule with caspsae-2 in the time courses of expression in retinal tissues. BDNF administered before reperfusion inhibited the expression of apoptosis and ameliorated the retinal tissue damage. It also decreased caspase-2 and caspase-3 expression in ischemic/reperfused retina.

• CONCLUSION: Retinal ischemia-reperfusion can induce apoptosis of cells in the retina. BDNF rescues retinal ganglion cells (RGCs) from retinal ischemia/reperfusion injury through down-regulation of cell apoptosis and caspase-2 and caspase-3 expression. BDNF have a neuroprotective effect on retina.

• KEYWORDS: retina; ischemia/reperfusion injury; apoptosis; brain derived neurotrophic factor; caspase-2; caspase-3

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INTRODUCTION

Retinal ischemia is a serious and common problem that occurs as a result of acute vascular occlusion and leads to loss of vision in a number of ocular diseases such as acute glaucoma, diabetic retinopathy, hypertensive retinopathy, and retinal vascular occlusion[1,2]. Recent studies have shown that most of the neuronal cell death associated with retinal ischemia-reperfusion injuries is due to apoptosis [3-5]. And caspase-2 and caspase-3 are the two important members of the caspase family and play different roles in the process of neuronal apoptosis.

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that suppresses neuronal death from various injuries [6-8]. Although little is known about how BDNF protects neurons

from such injuries, it is likely that it modifies some mechanisms in the pathway of cell death. In this study, the association between cell death after ischemia-reperfusion injury in the retinal ganglion cell layer (GCL) and the expression of caspase-2 and caspase-3 will be revealed, at the same time, the neuroprotective effects of BDNF will be also described.

MATERIALS AND METHODS

Animal and Reagents A total of 28 healthy wistar albino rats (body weight 250-300g, female 14 and male 14) were obtained from Animal Experimental Center of Qingdao and divided into Normal and Operative Groups randomly. In Operative Group, the left eyes were injected with normal saline into the vitreous cavity at 1 hour after ischemia (regarded as Ischemia/ reperfusion Group), and the right eyes were injected with BDNF (100ng) into the vitreous cavity at the same time (regarded as Operative Group). The Operative Group was subdivided into groups of 1, 6, 12,24, 48, 72 hours according to the time after reperfusion. Rabbit anti-rat caspase-2 and rabbit anti-rat caspase-3 polyclonal antibody and strept avidin-biotin complex (SABC) immunohistochemistry kit came from Boster Company, Wuhan. TUNEL kit, ELISA kit and recombinant human brain derived neurotrophic factor (BDNF, 5μg) were obtained from Jingmei Company, Beijing. The retinal ischemia/reperfusion injury rats model were made by transiently elevating intraocular pressure according to methods in literature [6]. A 5 intravenous needle was connected to a bottle of balanced saline solution (BSS) which was capable of produce a static pressure of 110mmHg.The needle was injected into the anterior chamber of the eyes through corneal limbus to make blood flow of central retinal artery stop. The initial IOP of 28 rats was between 15mmHg and 20mmHg. The ischemic procedure lasted for 60 minutes. The IOP was then adjusted to normal and the retinal blood flow was restored. The fundus of the eyes were observed through direct and indirect ophthalmoscope after the pupils were dilated with bistropamide .The interruption of blood flow of the retinal artery and paleness of bulbar conjunctiva demonstrated the successful establishment of retina ischemia.When the IOP was changed to normal, the retinal blood flow and the blood supply of conjunctiva restored, which marked the success of retinal ischemia/reperfusion injury.

***In Situ* Detection of Apoptotic Cells** Paraffin sections were dewaxed by xylene for 12 minutes at room temperature; hydrated by a graded alcohol series gradually, including 990, 950, 800, 750mL/L alcohol 5 minutes respectively, and then endogenous peroxidase was inactivated by covering the sections with 30mL/L H_2O_2 for 10 minutes at room temperature. After that, they were digested by protease for 5 minutes at room temperature. Sections were incubated with 20μL terminal deoxynucleotidyl transferase (TdT) in a moist chamber at 37℃ for 2 hours. Then 50μL biotin-anti-digoxin antibody was added on each section for 30 minutes at 37℃. SABC were added for 30 minutes at 37℃. The sections showed color by adding diaminobenzidine (DAB) and were slightly counterstained by hematein, then dehydrated, hyalined, sealed and observed under the optiacl microscope (BX-51,Olympus). The reaction was terminated by transferring the slides to phosphate buffer (PBS) at room temperature for 15 minutes. As a negative control, sections of retina were exposed to phosphate buffer solution(PBS) instead of TdT.

Immunohistochemistry Studies Paraffin sections were dewaxed, hydrated by a graded alcohol series gradually. They were covered with 30mL/L H_2O_2 for 10 minutes at room temperature and then digested by 1g/L protease for 5 minutes at room temperature. Normal goat serum were added on sections for 10 minutes at room temperature. Rabbit-anti-rat caspase-2 polyclonal antibody and rabbit-anti-rat caspase-3 polyclonal antibody were added in a humid chamber at 37℃ for 20 minutes. Then SABC was added for 20 minutes at room temperature; DAB was added on sections to show color. The sections were slightly counterstained by hematoxylin, then dehydrated, hyalined, sealed and observed under the microscocope. As a negative control, the sections were incubated with PBS instead of the primary antibody.

ELISA Standard protocol for caspase-2 and caspase-3 ELISA was followed to measure the levels of immunoreactive caspase-2 and caspase-3 in retina after I/R injury according to the manufacturer's instructions. Normal retinae and retinae at 1, 6, 12, 24, 48 and 72 hours after I/R injury (n =4 for each time point) were collected and homogenized for 30 seconds in 200μL ice-cold PBS (0.1mol/L, pH7.4). The homogenates were centrifuged for 10 minutes at 17 000g at 4℃, and 100μL of the supernatant was used for each determination. The optical density of each sample (in triplicate) were determined with a microplate spectrophotometer(HT340,Bio-TeK).Caspase-2 and caspase-3 contents was calculated according to the manufacturer's instructions and reported as picograms per millilitre. The assay was repeated, and the averages of the two runs were reported.

Result Observation Results were observed by optic microscope (BX-51,Olympus). TUNEL and immunohistochemistry positive cells were identified by nucleus stained yellow or yellow-brown. Sections were scanned by image analysis system (OPTONVIDAS); four sights were chosen

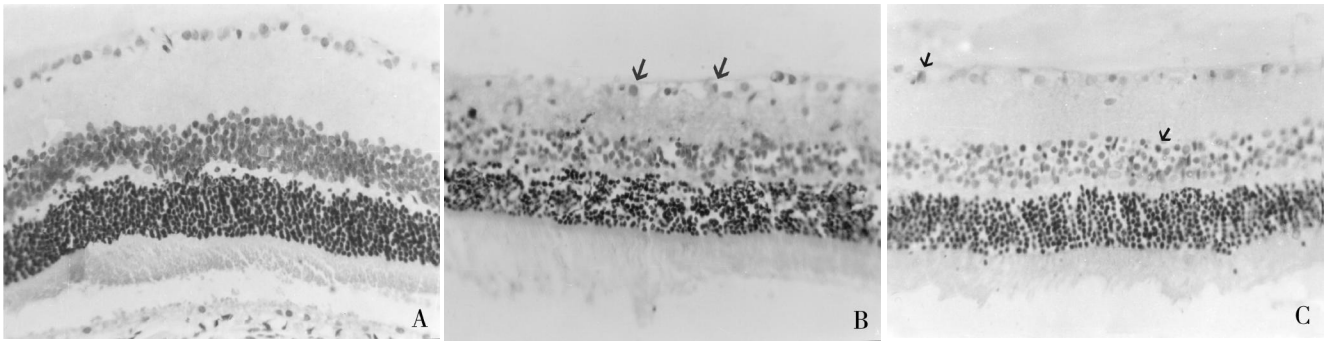

Figure 1 Apoptosis cells in rat retinal tissues (SABC×400) A: normal; B: I/R group; C: BDNF-treated group

from each section and each sight had an area of 0.2mm × 0.2mm. Every two sights were taken beside the optic nerve. Then the average positively stained cell quantity was obtained. ELISA result was calculated according to the manufacturer's instructions.

Statistical Analysis The data were analyzed with SPSS 10.0 software. Pair-matching test was used to compare the same time courses of two different groups. P <0.05 was taken to be statistically significant.

RESULTS

***In Situ* Detection of Atoptosis in Ischemic/reperfused Retina** There were no apoptotic cells in normal retinal tissues. At 6 hours, there were a small quantity of yellow-stained cells in GCL (aç l k şekliekli yaz1lmam1ş) in ischemic/reperfusion group; at 12 hours, apoptotic cells increased significantly; the number of apoptotic cells reached a peak at 24 hours and all layers were yellow-stained; at 48 hours, the number of apoptotic cells decreased; and at 72 hours apoptotic cells also could be found. In the BDNF treated group, there was a significant reduction in the number of apoptotic cells at 12, 24, 48 hours compared with Ischemia/reperfusion Group (Table1, Figure 1).

Immunohistochemistry There were no caspase-2 positive cells in normal retinal tissue. In the I/R, there were a small quantity of caspase-2 positive expressions at 6 hours; at 12 hours, the number of caspase-2 positive cells increased significantly; the expression reached the peak at 24 hours; at 48 hours the number of apoptotic cells decreased; and at 72 hours caspase-2 expression obviously descended. In the BDNF-treated Group, caspase-2 expressions changed similarly to those in the I/R except that the expression intensity obviously weakened at 6, 12 ,24 and 48 hours after reperfusion. Similarly, caspase-3 has a similar expression trend with caspsae-2 in the time of expression after reperfusion and the expression intensity obviously weakened at each time course in the BDNF-treated Group (Table 2, Figure 2).

Table 1 Comparison of numbers of apoptosis cells after ischemia/ reperfusion injury (n =4, mean ± SD, positive cells/mm^2)

t (h)	I/R	BDNF	t	P
1	5.6±0.9	4.7±0.9	1.4142	0.2070
6	479.5±98.6	329.9±48.1[a]	2.7273	0.0343
12	1097.6±88.1	846.7±11.9[a]	5.6445	0.0013
24	1344.5±121.9	976.7±89.8[a]	4.8585	0.0028
48	674.6±58.1	489.9±45.1[a]	5.0224	0.0024
72	69.9±19.0	68.3±32.4	0.0852	0.9349

Comparison of each treated group to its prior ischemic group within each time course: [a]P<0.05

Table 2 Caspase-2, caspase-3 expressions after ischemia/ reperfusion injury (n =4, mean ± SD, positive cells/m^2)

t(h)	Caspase-2		Caspase-3	
	I/R	BDNF	I/R	BDNF
1	3.9±0.8	2.7±0.4	2.0±0.7	1.9±0.6
6	89.9±10.7	71.0±8.9[a]	85.7±9.8	62.0±10.1[a]
12	289.0±31.9	230.0±29.7[a]	497.6±32.1	354.7±29.1[a]
24	708.5±45.4	587.2±35.1[a]	897.6±31.1	773.1±29.7[a]
48	522.2±24.6	480.3±19.3[a]	261.7±14.1	205.9±10.9[a]
72	161.7±16.6	143.2±14.1	45.1±5.4	38.9±2.9

[a]P <0.05 *vs* prior ischemic groups

ELISA In normal retinal tissues, there were no caspase-2 activity; in the I/R Group, there was a small content of caspase-2 in retinal tissue at 6 hours; at 12 hours, the content increased and the content reached the peak at 24 hours; at 48 hours, the caspase-2 content began to decrease; and at 72 hours the caspase-2 content obviously descended. In the BDNF-treated Group, caspase-2 expressions changed similarly to those of the I/R Group except that the content obviously decreased at 6, 12 and 24 hours after reperfusion. Similarly, caspase-3 has the same expression rule with caspsae-2 in the time courses of expression in retinal tissues and the content obviously decreased at each time course in the BDNF-treated Group (Table 3).

DISCUSSION

Apoptosis is an important process in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis

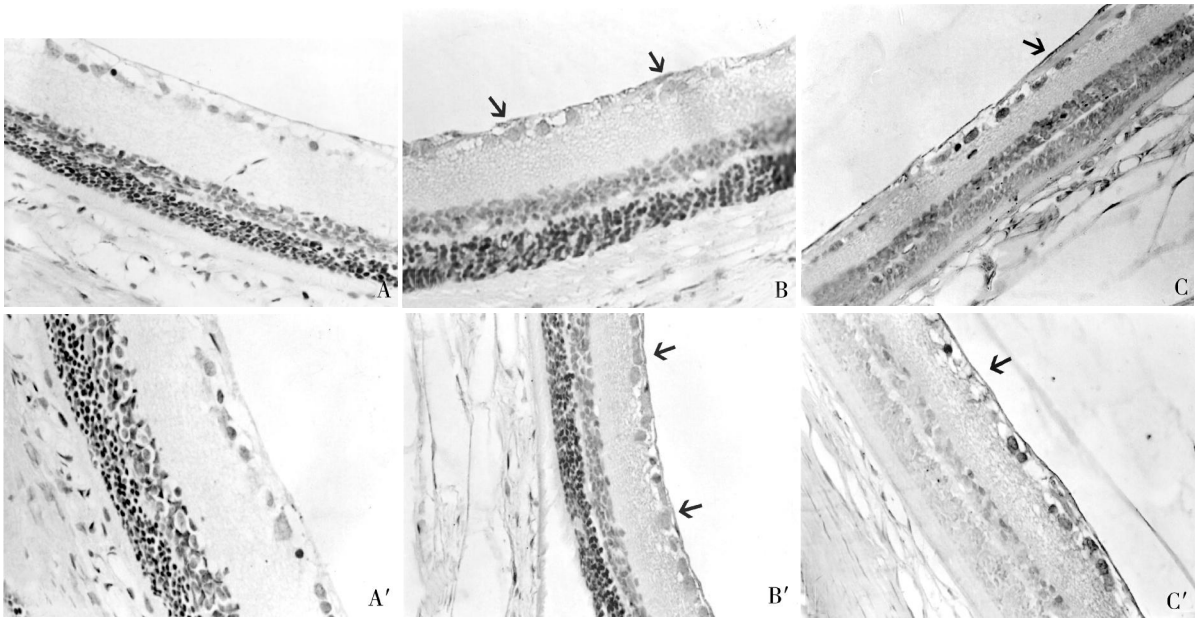

Figure 2 Positive cells for caspase-2, caspase-3 in rat retinal tissues (SABC×400) A/A′ : Normal; B/B′ : I/R group; C/C′ : BDNF-treated group; A,B,C for caspase-2, A′ , B′ , C′ for caspase-3

Table 3 Changes of caspase-2, caspase-3 contents after ischemia/ reperfusion injury (n =4, mean ± SD, ng/L)

t /h	Caspase-2		Caspase-3	
	I/R	BDNF	I/R	BDNF
1	27.8±3.0	26.7±2.9	46.9±4.1	47.5±5.0
6	90.5±10.5	85.6±11.6	107.9±11.1	94.6±10.1
12	196.7±20.6	160.3±12.7[a]	290.6±20.9	230.7±19.5[a]
24	251.9±30.1	189.6±17.6[a]	369.7±31.4	280.6±31.2[a]
48	125.6±10.9	100.5±8.9[a]	159.3±12.9	121.6±10.5[a]
72	54.5±6.7	49.7±6.0	82.1±7.6	77.5±9.0

[a]P<0.05 *vs* prior ischemic groups

and hormone dependent atrophy, and also in chemical-induced cell death. Apoptosis is characterized by condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, dilatation of the endoplasmic reticulum [9], a decrease in cell volume and alterations to the plasma membrane resulting in the recognition and phagocytosis of apoptotic cells, thus preventing an inflammatory response. TUNEL method can mark apoptotic cells *in situ* , which identified apoptosis during retinal ischemia/reperfusion injury reliably and directly. In present study, little apoptosis in GCL and INL layers before 12-hour ischemia/reperfusion was observed. Apoptotic cells reached to the peak after 24-hour ischemia/ reperfusion and decreased after 48-hour ischemia/ reperfusion gradually. Those studies proved that cells died mainly through apoptosis in the RIRI and in accordance with the research by Buchi and Kaneda *et al* [10]. It has been found that apoptosis is a complicated proteolytic enzyme cascade reaction course and caspases are important in the signaling pathway of cellular apoptosis [11,12]. Caspase-2 and caspase-3 are two important members of caspase family. Caspase-2, initially was denominated nedd22 /ich21; the activated caspase-2 can induce the release of apoptosis related protein directly or indirectly through cut bid (a sort of Bcl-2 protein which can promote the apoptosis to generate), including cyt2c, Smac (second mitochondrial apoptosis inducing factor) and AIF (apoptosis inducing factor). In caspase family caspase-2 has two functions:the first one is signal transmission function ,and throught it caspase-2 can activate its downstream caspase.Many researches have demonstrated that incorporating the structural domain of caspase-2 and proenzyme of caspase-3 can enhance the self-activation of zymogen and apoptosis induced by caspase-3 [12]. Second, caspase-2 can crack protein directly and result in apoptosis. During the execution phase of apoptosis, caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins; Caspase-3, a member of the CED-3 subfamily of caspases, is widely distributed, with high expression in cell lines of lymphocytic origin, suggesting that it may be an important mediator of apoptosis in the immune system[13]. In general, caspase-3 is a key enzyme for apoptotic cell death and is recognized as a terminator for cells to be killed[14].

Caspase-2 and caspase-3 expressions were markedly upregulated in GCL and NFL after RIRI. The expression time tendency of two caspases and apoptosis was similar. There were a small quantity of caspase-2 and caspase-3 positive expressions at 6 hours; the expression reached the peak at 24 hours and then decreased gradually after 48

hours. Again, the time courses of the apoptotic cell number and the contents of caspase-2 and caspase-3 detected by ELISA and immunohistochemistry showed a similar pattern. From those we could find that RIRI caused an increase in caspase-2 and caspase-3 expression and was in accordance with TUNEL in time and sites. Our data suggested, therefore, that overexpression of caspase-2 and caspase-3 might result in apoptosis in retina after ischemic/reperfusion.

In recent years, an increasing number of neurotrophic factors have been applied to clinic to rescue retinal function. Neurocyte under normal growth needs enough neurotrophic factors from its target tissue and/or colloid cell of distal end which will be abscised after nerve injury. RGC will die because of injury and dystrophy. But the injured RGC remain in better growth if ectogenic neurotrophic factors are given in time [15]. The biological actions of neurotrophic factors to nervous system can be summarized into two aspects: one is increasing the survival rate of neurocyte, called protective action; the other is facilitating the nervous process growth of neurocyte, called regenerative action. For instance, they can facilitate the regeneration of RGC axon and the extension of nerve fiber, induce the directional growth of axon and decide the growth direction of nerve fiber. Brain-derived neurotrophic factor (BDNF) is a neurotrophin that suppresses neuronal death from various injuries [16]. Many experiments suggest that BDNF can also promote the survival of RGC *in vitro*, accelerate its proliferation and promote the development of axon[17,18].

In our study, we found that brain derived neurotrophic factor (BDNF) intravitreous injection reduced the quantity of apoptotic cells in RIRI. In treatment group, the quantity of caspase-2 and caspase-3 positive cells decreased at 6, 12, 24 and 48 hours after reperfusion. Meanwhile, the results showed a similar pattern in TUNEL method. It suggested that BDNF has therapeutic effect on retina after ischemic/reperfusion.

In summary, we have shown that the expression of caspase-2 and caspase-3 are associated with cells death in the retinal GCL in a rat ischemia/reperfusion injury model. Our study provides further insight into the mechanisms of BDNF neuroprotection, one of which is that BDNF could partly contribute to its inhibition to apoptosis by its inhibition to caspase-2 and caspase-3 expression. Because they turns out to be the crucial executioner of apoptotic death in neurons, regulation of its activity promises to be a powerful tool in controlling neuronal cell survival. Whether retinal caspase-2 and caspase-3 activity, however, is under direct regulatory control of BDNF signal transduction or whether BDNF mediated inactivation of certain initiator caspases is subject to current investigation.

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