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GRGM-13 comprising 13 plant and animal products, inhibited oxidative stress induced apoptosis in retinal ganglion cells by inhibiting P2RX7/p38 MAPK signaling pathway

Qiu-li Zhang[a,*,1], Wei Wang[b,1], Yan Jiang[a], A-tuya[a], Dongmei[a], Lan-lan Li[a], Zhan-Jun Lu[a], He Chang[a], Tian-Zi Zhang[a,*]

[a] Department of Ophthalmology, Affiliated Hospital of Inner Mongolia University for the Nationalities, No 1742, HuoLinHe Street, Neimenggu, Tongliao, 028050, China
[b] Department of Ophthalmology, Tongliao Hospital, No.668, KeErQin Street, Neimenggu, Tongliao, 028000, China

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ABSTRACT

Objective: To determine the effectiveness of GRGM-13 on oxidative stress induced apoptosis of retinal ganglion cells (RGCs) and revealed its possible mechanism.

Materials and methods: Caspase-3 activity, MDA level, and glutathione peroxidase level were detected by Caspase-3 assay kit, Lipid Peroxidation MDA Assay Kit, and Total Glutathione Peroxidase Assay Kit, respectively. Protein levels of Bax, Bcl-2, p-p38 and p38 were observed by Western Blot. Reactive oxygen species assay kit was used to determine intracellular ROS level. Apoptotic cells were measured by flow cytometry.

Results: GRGM-13 inhibited apoptosis of RGCs and ROS level in rat retinal tissue and RGC-5 cells, and the decrease degree strengthened with the increase of GRGM-13 concentration. In addition, ROS upregulated p-p38 expression, while GRGM-13 reversed this effect. We also found that p38 inhibitor SB202190 did not change L-glutamate (Glu) or H_2O_2-induced ROS level, while SB202190 inhibited apoptosis of RGC-5 cells. Finally, we observed that P2 × 7R agonist BzATP reversed the inhibition effect of GRGM-13 on RGC-5 cell apoptosis, ROS level and p-p38 expression, while si-P2 × 7R inhibited oxidative stress-induced phosphorylation of p38.

Conclusion: GRGM-13 could inhibit oxidative stress-induced RGCs apoptosis via inhibiting P2RX7/p38 MAPK pathway, which revealed the possible mechanism of GRGM-13 on stress-induced RGCs apoptosis and provided new Chinese medicine for the treatment of glaucoma.

1. Introduction

Glaucoma is a complicated disease characterized by high intraocular pressure, pain, and retinal ganglion cell (RGCs) degeneration, which causes irreversible visual impairment and blindness [1,2]. In 2010, about 60.5 million people in the world are affected by primary angle-closure glaucoma (PACG) and primary open-angle glaucoma (POAG), and the prevalence of PACG is highest in Asia and the prevalence of POAG is highest in Africa [3]. Glaucoma is growing fast and may increase to 76.0 million in 2020, which brings huge burden to patients and families [3]. Therefore, it is important to find effective strategies to prevent glaucoma. It has been reported that with the increase of age, the amount of RGCs was decreased [4]. Researches have revealed possible mechanisms of RGC death in glaucoma, such as oxidative stress, mitochondrial dysfunction, activation of extrinsic and intrinsic apoptotic pathways, etc. [5]. In addition, Chi et al found oxidative stress and neuro-inflammation were vital for the pathogenesis for RGC death during acute glaucoma [6]. Hence, this study focused on the exact mechanism of oxidative stress induced RGCs apoptosis.

Gurigumu-13 (GRGM-13) is a commonly used Mongolian and Tibet traditional medicine which is composed of thirteen individual traditional medicines, safflower (Flos carthami), clove (Syzygium aromaticum), lotus seed (Nelumbo nucifera), liriope (Ophiopogon japonicus), radix aucklandiae (Saussurea lappa), fructus chebulae (Terminalia chebula Retz), chinaberry fruit (MeLia toosendan Sieb), gardenia (Gardenia jasminoides Ellis), lignum pterocarri (Pterocarpus santalinus), artificial musk (Moschus), pulvis cornus bubali concentratus (Bubali Cornu), bovis calculus artifactus (Bos taurus domesticus Gmelin) and vermilion. Xu et al have proved that Gurigumu have hepato-protective effects, which increased superoxide dismutase activity and decreased malondialdehyde (MDA) level [7]. Safflower, a main component of GRGM-13, can clear liver warm. Hydroxysafflor

* Corresponding authors.
E-mail addresses: qiu_lizhang@126.com (Q.-l. Zhang), z_tianzi@163.com, ztzhlk6646@126.com (T.-Z. Zhang).
[1] These authors contributed equally to this work.

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yellow A (HSYA), the major water-soluble component in safflower [8], has been proved to reduce cardiovascular and liver injury caused by oxidative stress [9]. Liu et al reported that HSYA suppressed liver fibrosis by inhibiting p38 MAPK signaling [10]. In addition, Takayuki et al found that p38 MAPK participated in the progress of RGCs survival [11]. Moreover, researchers have revealed that purinergic P2 × 7 receptor (P2 × 7R) controlled reactive oxygen species (ROS) generation, and proved that P2 × 7R stimulated phosphorylation of p38 MAPK [12]. Therefore, we speculated that Gurigumu-13 inhibited P2 × 7R, thus inhibited ROS generation to further inhibit phosphorylation of p38, and finally inhibited RGCs apoptosis.

In this study, we aimed to determine whether GRGM-13 could inhibit oxidative stress-induced RGCs apoptosis, and to figure out the exact mechanism of oxidative stress induced RGCs apoptosis. We measured the activity of caspase-3, expressions of apoptosis related proteins (Bax and Bcl-2), MDA level and glutathione peroxidase level in rat retinal tissue among different treated groups. Also, we detected apoptosis rate and cellular ROS level of RGCs. Finally, we discovered that P2 × 7R/p38 MAPK participated in the inhibition of GRGM-13 on RGCs apoptosis.

2. Materials and methods

2.1. Chronic ocular hypertension (COH) rat model

Thirty male Sprague Dawley (SD) rats weighing about 300–350 g were purchased from Laboratory Animal Center of Jilin University, and kept in a standard animal room under a cycle of 12 h of light and 12 h of darkness (from 6:00 A.M. to 6:00 P.M.) with free access to food and water. On the day of the surgery, rats were anesthetized using 15 mg/kg pentobarbital sodium (Sinopharm Chemical Reagent Co., Shanghai, China) with intraperitoneal administration, and the left eye was injected with 1.8 M NaCl via an episcleral vein. The right eye was used as the control eye without treatment. A microglass needle was inserted into the episcleral vein, and hypertonic saline (50 μl) was injected into the left eye. This procedure produced scarring of the trabecular meshwork, and 7 to 10 days later, there was a rise in increased intraocular.

To determine the levels of intraocular pressure, rats were sedated with 2mg/kg acepromazine through intramuscular injection, and intraocular pressure was measured by a Tonolab tonometer 5 min after injection. Both eyes were measured ten times and the average readings were obtained.

After the administration, rats were sacrificed to prepare the retinal tissue. Under the microscope, the eyeball was cut along corneal limbus, the cornea, crystalline lens and vitreous body were removed, and the retinal tissue was stripped. Retinal tissue was washed with pre-cooling deionized water, the filter paper was used to dry the water, and finally retinal tissue was placed in frozen pipes.

GRGM-13 pills were purchased from Tongrentang (Tongliao, China). The contents of GRGM-13 pills were Flos carthami (60 g), Syzygium aromaticum (30 g), Nelumbo nucifera (30 g), Ophiopogon japonicus (60 g), Saussurea lappa (30 g), Terminalia chebula Retz (30 g), MeLia toosendan Sieb (30 g), Gardenia jasminoides Ellis (30 g), Pterocarpus santalinus (30 g), artificial musk (0.5 g), pulvis cornus bubali concentratus (30 g), bovis calculus artifactus (30 g) and vermilion (30 g). SD rats were divided into five groups and each group consisted of six rats: control group, chronic ocular hypertension (COH) group, COH + low dose of GRGM group (0.4 g/kg/d), COH + medium dose of GRGM group (0.8 g/kg/d), COH + high dose of GRGM group (1.6 g/kg/d). Rats in the five groups were treated by intragastric administration of distilled water, distilled water, 0.4 g/kg/d GRGM, 0.8 g/kg/d GRGM and 1.6 g/kg/d GRGM, respectively, with a volume of 2 mL, once a day for nine days. All animal experiments were approved by the ethics committee of inner Mongolia University for the nationalities.

2.2. Preparation of serum containing GRGM

Twenty male SD rats were divided into two groups: serum containing GRGM-13 group (n = 10) and normal group (n = 10). Serum containing GRGM-13 rats were obtained by intragastric administration of GRGM (8.4 g/kg/d, which corresponds to 10 times the adult clinical dose). The intragastric frequency was two times a day (at least 6 h at intervals) for 3 days. The blood was taken from the abdominal aorta 2 h after the last GRGM-13 administration. The serum was isolated and stored at −20℃. Rats in control serum group were treated with equal volume of distilled water.

2.3. Cell culture and small interfering RNAs transfection

The purpose of exposing RGC cells to si-RNA transfection was to observe the effect of P2RX7 on p38 phosphorylation. RGC-5 cell line was purchased from American Type Culture Collection (ATCC), and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% fetal bovine serumin (FBS, Invitrogen, USA) and 100μg/mL streptomycin (Invitrogen, USA) in 5% CO_2 incubator under 37℃. Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, USA) was used for siRNA transfection according to the manufacturer's instructions. P2 × 7R siRNA sequence: 5'-AACCAGAG GAGAUACAGAUTT-3'.

2.4. Measurement of caspase-3 activity

Caspase-3 assay kit (Sigma, USA) was used to measure the caspase-3 activity according to the manufacturer's instructions. The base of this kit was the hydrolysis of the peptide substrate acetyl-Asp-Glu-vial-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3 which resulted in the release of the p-nitroaniline (pNA) moiety. Homogenate of rat retinal tissue lysed with chilled lysis buffer and incubated on ice for 10 min, then centrifuged at 12,000 rpm for 5 min at 4 ℃. The supernatant was collected and combined with an equal amount of substrate reaction buffer containing a caspase-3colorimetric substrate. After incubation at 37 ℃ for 2 h, the pNA light emission was quantified using a microplate reader at 405 nm (BioTek, USA). The percentage of the increase in caspase-3 activity was compared with the absorbance of pNA from control sample [13].

2.5. Western blot analysis

RIPA buffer (Invitrogen, USA) was used to extract proteins from rat retinal tissues and RGC-5 cells from different groups. The concentration of sample protein has been determined by Brandford protein Kit (Beyotime Biotechnology, China). Sample proteins (contained same amount of proteins) were separated on 10% SDS-polyacrylamide gel electrophoresis ((SDS-PAGE)) and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, USA), including with primary antibodies anti-Bax (diluted at 1 : 1000, Invitrogen, USA), anti-Bcl-2 (diluted at 1 : 1000, Merck Millipore, USA), anti-α-Tubulin (diluted at 1 : 1000, Sigma, USA), anti-p-p38 (diluted at 1 : 1000, Invitrogen, USA), anti-p38 (diluted at 1 : 1000, Invitrogen, USA), anti-β-actin (diluted at 1 : 1000, Invitrogen, USA). The corresponding horseradish peroxidase-conjugated secondary antibody (Invitrogen, USA) was added and bands were visualized by Typhoon 9410 (GE Healthcare, USA).

2.6. Determination of intracellular ROS

Reactive oxygen species assay kit (Beyotime, Nantong, China) was used to determine intracellular ROS according to the manufacturer's instructions. RGC-5 cells were subjected to Glu, GRGM, or N-acetyl-L-cysteine (NAC, 5 mM), and incubated with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (10 μM/L) at 37 °C for 20 min. Then, cells were washed with serum-free medium for three times. Fluorescence intensity