Original Article
Tanshinone II-A protects retinal ganglion cells against retinal ischemia/reperfusion injury

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Abstract: We aimed to evaluate the protective effects of tanshinone II-A (TanIIA) on retinal ischemia and reperfusion (I/R) injury. The primary retinal ganglion cells (RGCs) were isolated from the retinae of Sprague-Dawley (SD) rats and then were induced retinal I/R injury in vitro. The RGCs subjected to I/R injury were treated with different concentrations of TanIIA (1.0 mM, 2.5 mM or 5.0 mM, respectively). Cell viability, Caspase-3 activity, LDH release, cell apoptosis and the level of mitochondria-derived ROS of different treatment groups were analyzed, respectively. Additionally, the expression levels of key molecules involving in Akt/MAPK signaling pathway, such as Akt, p-Akt, mTOR, p-mTOR, MAPK and p-MAPK, and apoptotic proteins, like Bcl2 and Bax, were determined by western blot. TanIIA treatment promoted cell viability and decreased caspase-3 activity and LDH release in a dose-dependent manner. What's more, TanIIA treatment inhibited cell apoptosis and the production of mitochondria-derived ROS in a dose-dependent manner. Besides, TanIIA treatment resulted in a markedly decrease in the expression of key proteins involving in Akt/MAPK signaling pathway, such as Akt, p-Akt, mTOR, p-mTOR, MAPK and p-MAPK, as well as the expression of apoptotic protein Bax. Our findings indicate that TanIIA treatment may promote cell viability and inhibit cell apoptosis after cells were induced retinal I/R injury. Moreover, TanIIA may protect RGCs against retinal I/R injury via inhibiting cell apoptosis or suppressing Akt/MAPK signaling pathway. TanIIA may serve as a potential therapeutic reagent for the treatment of ocular diseases.

Keywords: Tanshinone II-A, ischemia/reperfusion injury, Akt/MAPK signaling pathway, cell viability, cell apoptosis

Introduction
Ischemia and reperfusion (I/R) injury is considered as a common cause of several vascular and neuronal diseases, such as ischemic stroke and myocardial infarction [1]. In comparison with other tissues, the retina is more vulnerable to I/R injury due to the highest metabolism and oxygen consumption of it [2]. Retinal ischemia depletes the delivery of oxygen and essential nutrients and results in cell death, and the subsequent reperfusion, although seemingly helpful to restore this disrupted retinal circulation, can aggravate ischemic injury and further cause inflammation [3, 4]. Many ocular diseases, including ischemic optic neuropathy, retinal vascular occlusions, and acute glaucoma, are shown to be associated with retinal I/R injury [5]. Therefore, understanding the mechanisms involving in retinal I/R injury will facilitate to investigate effective therapeutic strategies for preventing such diseases.

Danshen, a dried root of Salvia miltiorrhiza, is the most popular medicinal herbs used in China and tanshinone II-A (TanIIA) is proved to be one of pharmacologically active components of danshen [6, 7]. TanIIA has been shown to have anti-cancer effects in a variety of cancer cell types. For instance, TanIIA could inhibit cell growth, suppress invasion and induce apoptosis in human hepatocellular carcinoma cells [8, 9]. In human colorectal cancer, TanIIA can effectively inhibit angiogenesis via down-regulating cyclooxygenase-2 [10]. In addition, the cardioprotective effects of TanIIA on the cardiovascular system have attracted great interest [11]. TanIIA is able to protect the cardiomyocytes against the adriamycin-induced myocardial injury in rat model via preventing the activation of cardiac fibrosis and apoptosis [12]. Besides, TanIIA has been widely used in the treatment of I/R injury. Pharmacological postconditioning with TanIIA protects the myocardium from I/R injury through activating the phosphatidylinositol 3-kinase pathway [13].
TanIIA is also shown to have neuroprotective capabilities against cerebral I/R injury via anti-apoptotic pathway in rats [14]. However, to the best of our knowledge, the neuroprotective effects of TanIIA against retinal I/R injury have not been fully investigated.

In the current study, we constructed retinal I/R injury model using primary retinal ganglion cells (RGCs). Cell viability assay, caspase-3 activity detection, LDH release assay, in situ nick end-labeling (TUNEL) assay and mitochondria-derived ROS detection were respectively performed to evaluate the protective effects of TanIIA on retinal I/R injury. Besides, the expression of apoptotic proteins or key proteins involved in Akt/MAPK signaling pathway was determined by western blot, thus to elucidate the possible mechanism of TanIIA in preventing retinal I/R injury.

Materials and methods

Animals

All animal experimentation was performed in conformity with the Guidelines of the Care and Use of Laboratory Animals and approved by our Institute’s Animal Ethics Committee. Adult Sprague-Dawley (SD) rats (weighing 250-300 g) were originally purchased from Harlan Laboratories and were housed in a specific pathogen-free animal room under a 12-hour light-dark cycle with access to standard food and water. Animals were then humanely sacrificed with the treatment of CO$_2$ exposure.

Isolation and cultivation of RGCs

Retinae were dissected from SD rats as previously described and were then dissociated enzymatically using the Papain Dissociation Kit (Worthington Biochem, New Jersey, USA) to obtain a mixed suspension of RGCs. The number of dissociated RGCs was determined by trypan blue test, and then RGCs ($5 \times 10^5$ viable cells per well) were plated on 13 mm diameter coverslips (VWR, Lutterworth UK). RGCs were cultured in B27-supplemented Neurobasal-A medium (Invitrogen Inc., Carlsbad, CA, USA) supplemented with L-glutamine and gentamicin for 6 d at 37°C in a humidified chamber with 5% CO$_2$. These experiments were repeated 3 times at least.

In vitro I/R injury

In vitro retinal I/R injury was induced as previously described with slight modifications [15]. Briefly, RGCs were putted into the 37°C incubator with the Dulbecco Modified Eagle Medium (DMEM) lacking glucose and FBS under ischemic conditions: (1% O$_2$ and 5% CO$_2$). After 2 h of ischemic treatment, the cells were changed to culture in complete media under the normal culture condition for 12 h, which was considered as reperfusion. Cells were then harvested for subsequent analysis.

Cell treatment

TanIIA was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). TanIIA was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) for making a TanIIA stock solution and diluted to working concentration immediately before use. The RGCs subjected to I/R injury was treated with different concentrations of TanIIA (1.0 mM, 2.5 mM or 5.0 mM, respectively). Thus, the cultured RGCs were randomly divided into different groups: I/R group, I/R + TanIIA-1.0 group, I/R + TanIIA-2.5 group, I/R + TanIIA-5.0 group and control group. In the control group, RGCs were incubated under normoxic conditions for equivalent durations with the high-glucose DMEM.

Cell viability assay

Primary RGCs ($5 \times 10^4$ cells/mL) were seeded into 96-well plates and were incubated at 37°C in a humidified chamber with 5% CO$_2$ until 80% confluent. After the designated treatment, the cell viability of different groups was determined by MTT assay using a cell enzyme activity kit (Sigma, St. Louis, MO, USA). The optical density at 490 nm was measured to analyze the cell viability using a microplate reader (Model 550; Bio-Rad, Hercules, CA, USA). The cell viability of control cells was defined as 100%. Each experiment was performed in triplicate.

Caspase-3 activity

According to the manufacturer’s protocol, the activity of caspase-3 was determined by a colorimetric activity assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). In brief, RGCs
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were firstly lysed in ice-cold lysis buffer completely and then centrifuged at 4°C at 12000 g for 15 min. The equal amount of supernatant was then incubated with the caspase-3 substrate (Ac-DEVD-pNA) on a 96-well-plate. Finally, caspase-3 activity was determined using a microplate reader (Model 550; Bio-Rad, Hercules, CA, USA) at 405 nm.

**LDH release assay**

Cytosolic LDH release was used to quantitatively assess the cell injury using a cytotoxicity detection kit. In brief, cells were continuously exposed to 0.1% Triton X-100 to cause near complete glial cell death (100%), which was regarded as the maximal LDH value. Absorbance at 492 nm was measured to analyze then LDH values using a microplate reader (Model 550; Bio-Rad, Hercules, CA, USA). LDH release values were normalized to the mean maximal LDH value and expressed as a percentage of the maximal LDH level. Each experiment was repeated 3 times.

**TUNEL assay**

Apoptotic RGCs were detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method using ApoTag Peroxidase In Situ Apoptosis Detection Kit (S7100; Chemicon International, Billerica, MA, USA) following the instruction of the manufacturer. Cells were stained with diaminobenzidine (DAB; Santa Barbara, CA, USA) and then counterstained with methyl green. TUNEL-positive (TUNEL+) cells were then counted under an Olympus BX60 microscope (Tokyo, Japan, 400 × magnification) equipped with a digital charge-coupled device.

**Detection of mitochondria-derived ROS**

The level of mitochondrial superoxide production was detected using dichlorohydrofluorescein diacetate (DCFH) staining as the instruction of the manufacturer. In briefly, the cells in different treatment groups were washed once with phenolred-free medium. Then cells were incubated with 200 mL of DCFH-DA (10 mmol/L) at 37°C for 30 min. The fluorescence of DCFH in cells at the excitation and emission wavelengths of 485 nm and 530 nm represented the levels of mitochondria-derived ROS and was monitored using a fluorescence microscope (Olympus BX60, Tokyo, Japan).

**Western blot analysis**

Cells were firstly lysed with RIPA buffer containing protease inhibitors (BestBio, Shanghai, China) on ice completely. The concentration of protein was examined by Bradford (Bio-Rad, Madrid, Spain). Equal amounts of whole protein (30 ug) were separated on a 12% sodium dodecysulfate-polycrylamide (SDS-PAGE) gel. The fractionated proteins were then electrophoretically transferred to a nitrocellulose membrane (Millipore, Bedford, MA). After being blocked, the members were probed with primary antibodies, such as rabbit polyclonal antibodies against Akt (Cell Signaling Technology, Beverly, MA, USA), p-Akt (S473, Cell Signaling Technology), mTOR (Cell Signaling Technology), p-mTOR (Ser2448, Cell Signaling Technology), MAPK (Cell Signaling Technology), p-MAPK (Thr 202/Tyr 204, Cell Signaling Technology), Bcl2 (Pharmingen, San Diego, CA, USA), Bax (Pharmingen) and GAPDH (Santa Cruz Biotech, Delaware Avenue, CA, USA) overnight at 4°C. The membranes were then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotech) at room temperature for 2 h. The blots were visualized with enhanced chemiluminescence (ECL)-Plus reagent (GE Healthcare, Piscataway, New Jersey, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal loading control for measuring the expression level of these proteins.
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Statistics analysis

All data were expressed as Mean ± Standard Error of Mean (SEM). The normal distribution was tested using one-sample K-S test. Statistical analysis was performed with SPSS 20.0 or GraphPad Prism 5. Statistical significance was analyzed using the single-factorial analysis of variance (ANOVA) or Student’s t-test. Further comparison between groups was determined using post-hoc Tukey test. Statistical significance was considered when \( p \) values were less than 0.05.

Results

TanIIA treatment promoted cell viability in a dose-dependent manner

A MTT assay was performed to analyze the cell viability after drug treatments. As presented in Figure 1, the cell viability of I/R group was significantly decreased in comparison with control group (\( P < 0.05 \)). Compared with I/R group, we found that the cell viability was significantly promoted with the increasing concentration of TanIIA treatment (\( P < 0.05 \)), indicating that, during I/R injury, TanIIA treatment promoted cell viability in a dose-dependent manner.

TanIIA treatment decreased caspase-3 activity and LDH release in a dose-dependent manner

Figure 2 showed caspase-3 activity and LDH release after TanIIA treatment, respectively. The results showed that, after retinal I/R injury, caspase-3 activity and LDH release were significantly increased compared with control group (\( P < 0.05 \)). However, in comparison with I/R group, caspase-3 activity and LDH release were markedly decreased with the increasing concentration of TanIIA treatment (\( P < 0.05 \)), indicating that, during I/R injury, TanIIA treatment decreased caspase-3 activity and LDH release in a dose-dependent manner.

TanIIA treatment inhibited I/R-induced apoptosis in a dose-dependent manner

TUNEL staining displayed apoptotic cells of each group (Figure 3). Compared with control group, the quantity of apoptotic cells was significantly increased after retinal I/R injury (\( P < 0.05 \)). Notably, the quantity of apoptotic cells was markedly decreased with the increasing concentration of TanIIA treatment, when compared with I/R group (\( P < 0.05 \)). This result indicated that TanIIA treatment inhibited I/R injury-induced cell apoptosis in a dose-dependent manner.

TanIIA treatment inhibited the production of mitochondria-derived ROS in a dose-dependent manner

DCFH staining displayed the level of mitochondrial superoxide production in different treatment groups (Figure 4). Similar results were obtained that, with the increase of TanIIA dose, the production of mitochondria-derived ROS was significantly decreased compared with I/R group (\( P < 0.05 \)).
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TanIIA treatment may protect RGCs from I/R injury via suppressing Akt/MAPK signaling pathway or inhibiting cell apoptosis

In order to investigate the possible mechanism that TanIIA protected RGCs from I/R injury, western bolt was used to detect the expression of key molecules involving in Akt/MAPK signaling pathway and critical apoptotic proteins. The results showed that the expression of key molecules involving in Akt/MAPK signaling pathway, such as Akt, p-Akt, mTOR, p-mTOR, MAPK and p-MAPK was significantly decreased with the increasing concentration of TanIIA treatment, when compared with I/R group (P < 0.05, Figure 5A), implying that the Akt/MAPK signaling pathway may be key mechanism of TanIIA protection. In addition, similar results were obtained that, in comparison with I/R group, the expression of apoptotic protein Bax was markedly decreased with the increasing concentration of TanIIA treatment. On the other hand, TanIIA treatment obviously enhanced the expression of Bcl2 compared with I/R group and the expression was not changed significantly with the increasing concentration of TanIIA treatment; thereby the ratio of Bcl2 to Bax was increased by TanIIA in comparison with I/R group.

Discussion

The present study evaluated the probable protective effects of TanIIA on the retinal I/R injury.
We found that TanIIA markedly reduced retinal I/R injury via promoting cell viability and inhibiting cell apoptosis. TanIIA significantly decreased caspase-3 activity, LDH release and the production of mitochondria-derived ROS in a dose-dependent manner. In addition, TanIIA treatment resulted in a markedly decrease in the expression of key proteins involving in Akt/MAPK signaling pathway as well as the expression of apoptotic protein Bax. All these findings imply the protective effects of TanIIA on the retinal I/R injury and may merit further discussion.

In our study, TUNEL staining displayed that TanIIA significantly inhibited I/R-induced apoptosis in a dose-dependent manner. Apoptosis is an important biological mechanism in the process that cells adapt to new environmental changes. Cumulative evidence suggests that apoptosis after cerebral I/R injury is a major mechanism leading to the process of cell death [16]. As we all know, apoptosis can be regulated by a substantial body of regulatory genes mediating the apoptosis signals. Caspase-3, a central regulator of apoptosis, has been demonstrated to be up-regulated and activated in ischemic brain tissue [17]. Inhibition of caspase-3 activity is a key mechanism in the process that TanIIA protects against hypoxia-induced mitochondrial apoptosis [18]. Caspase-3 activity is also observed increased after myocardial cells being treated with angiotensin II, and TanIIA treatment can markedly attenuate angiotensin II-induced apoptosis via inhibiting the elevation of caspase-3 activity [19]. In our study, TanIIA treatment decreased the retinal I/R-elevated ecaspase-3 activity. It can therefore be speculated that our finding parallels these reports that caspases-3 activity is significantly increased when cells were subjected to I/R, and TanIIA treatment can reduce the magnitude of this increase. In addition, increasing evidence has suggested that an increase in the ratio of Bcl2 to Bax can prevent the progression of I/R-induced apoptosis in the myocardium [20]. In our study, the ratio of Bcl-2 to Bax markedly decreased after cells were subjected to I/R, while TanIIA treatment led to the increase in the ratio of Bcl2 to Bax, implying that the protective effects of TanIIA against retinal I/R injury are mediated by an increase in the ratio of Bcl2 to Bax. Taken together, we speculate that TanIIA may prevent I/R-induced RGCs from apoptosis via regulating caspases-3 activity and the ratio of Bcl2 to Bax, thus to play a protective effects on retinal I/R injury.

Furthermore, cerebral I/R injury is always associated with alterations of signal transduction pathways mediating inflammation and cell apoptosis [21]. Several studies have indicated that PI3K/Akt pathway and MAPK/ERK pathway play pivotal function in regulating cell death and survival [22, 23]. To investigate the key pathways involved in the protective effects of TanIIA on retinal I/R injury, we detected the expression of key proteins involving in Akt/MAPK signaling pathway, such as Akt, p-Akt, mTOR, p-mTOR, MAPK and p-MAPK. Akt, a primary mediator of the downstream effects of PI3K,
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can regulate cell proliferation and survival, and activation of the PI3K/Akt signaling pathway can protect against myocardial injury after transient cardiac ischemia [24]. It is reported that PI3K/Akt pathway can mediate the protective effect of humanin against cerebral I/R injury [25]. In addition, MAPKs are also reported to be activated after cerebral I/R, thus to play crucial roles in regulating neuronal survival or damage [26]. p38 MAPK and ERK signaling were also shown to be the key mechanism involved in the process that c-phycocyanin protects against I/R injury of heart [27]. Besides, pharmacological intervention of MAPK signaling pathway is likely to reduce apoptosis and inflammatory mediators, thereby playing a pivotal role in neuroprotection [28]. In our study, TanIIA treatment led to a markedly decrease in the expression of key proteins involving in Akt/MAPK signaling pathway. Therefore, we speculate that TanIIA may protect against retinal I/R injury via involvement of Akt/MAPK signaling pathway.

In conclusion, our results indicate that TanIIA treatment may promote cell viability and inhibit cell apoptosis after cells were induced retinal I/R injury. Moreover, TanIIA may protect RGCs against retinal I/R injury via inhibiting cell apoptosis or involvement of Akt/MAPK signaling pathway. TanIIA may serve as a potential therapeutic reagent for the treatment of ocular diseases.

Disclosure of conflict of interest

None.

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References


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