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Thioredoxin-Interacting Protein (TXNIP) Suppresses Expression of Glutamine Synthetase by Inducing Oxidative Stress in Retinal Muller Glia Under Diabetic Conditions

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Diabetic retinopathy (DR) is a progressive neurodegenerative disease with early-stage symptoms such as dysfunction of Muller cells, which leads to ganglion cell death. Its pathogenesis is probably associated with oxidative stress and a recently discovered protein, thioredoxin-interacting protein (TXNIP).

Material/Methods: To explore the role of TXNIP in DR, we cultured Muller cells under diabetic conditions, and then used immunohistochemistry, Western blot, and RT-PCR to detect the expression level of TXNIP under diabetic conditions. We demonstrated the expression level of glutamine synthetase (GS) when TXNIP was inhibited. To explore the potential pathway of TXNIP-induced cell damage in DR, we confirmed the role of IL-1β under diabetic conditions.

Results: Diabetes induces TXNIP expressions at mRNA levels, but shows the opposite effect on GS. IL-1β plays an important role in this pathway. Azaserine effectively increased the expression of GS via attenuating the expression of TXNIP.

Conclusions: This study demonstrates the role of TXNIP and its mechanism in DR, provides a possible treatment for DR, and lays a new theoretical foundation for the clinical treatment of DR and other diabetic microvascular changes.

MeSH Keywords: Diabetic Retinopathy • Interleukin-1beta • Oxidative Stress • Thioredoxins

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Background

Diabetic retinopathy (DR) is a progressive neurodegenerative disease, with early-stage symptoms such as dysfunction of Muller cells, which leads to ganglion cell death. Its pathogenesis is probably associated with oxidative stress, which promotes the production of reactive oxygen and nitrogen species (ROS/RNS) and leads to a vicious cycle of macromolecular damage. However, the details of this process are not entirely understood [1]. The incidence of diabetes has increased in recent years. There were 170 million people with diabetes in 2000, and the number of diabetics is expected to increase to 360 million by 2030 [2]. The microvasculature of diabetic patients is severely damaged through complex molecular mechanisms that are accompanied by serious complications, such as diabetic retinopathy (DR) [3]. About 43.1% of diabetic patients have DR [4], with symptoms of retinal hemorrhage, exudation, and traction retinal detachment, which are the most serious causes of blindness [1,5]. The lack of effective measures for treatment and prevention of DR leads to serious economic and emotional burdens to individuals, families, and society.

Recent studies found that DR is a neurodegenerative disease with a rapidly progressive course [6]. The pathological microenvironment of diabetes (e.g., hyperglycemia and hypoxia) causes abnormal structure and dysfunction of retinal cells, including retinal ganglion cell apoptosis, retinal vascular endothelial cells disorders, and abnormal metabolism in Muller cells [7]. There are 10 layers in the retina, in which Muller cells are the primary glial cells, playing important roles in maintaining the stability of the retinal environment. Early pathologies of DR, such as Muller cell dysfunction, directly lead to the apoptosis of ganglion cells [8]. The latest international studies tend to regard DR as "diabetic retinitis", indicating that inflammatory damage, including a series of processes such as oxidative stress, apoptosis, and cell proliferation, is the key to the pathogenesis of DR. However, the complex etiology makes prevention and treatment of DR difficult.

Our previous studies found that early pathologies of DR involve glutamate-mediated oxidative stress. High glucose levels cause disorders of the glutamate system, resulting in increased concentration of glutamate [9], in which glutamine synthetase (GS) is of crucial importance. However, our previous work failed to clarify the pathways and the relationships between upstream and downstream proteins. The recently discovered thioredoxin-interacting protein (TXNIP) can cause damage in oxidative stress via activating the reactive oxygen system, which induces cell death [10], but the exact pathways are still unclear. In a previous study, we found that GS plays an important role in glutamate-mediated oxidative stress, and the level of GS in Muller (retinal glial) cells was decreased under conditions with high glucose levels. Therefore, we hypothesized that TXNIP could initiate an oxidative stress reaction resulting in decreased GS, which leads to irreversible damage of ganglion cells in DR.

To further explore the role of expression of TXNIP in DR, as well as the relationship between TXNIP and GS, we designed the following experiments. In this study Muller cells were cultured in a high-glucose environment. Then immunohistochemistry, Western blot, and RT-PCR were used to detect the expression level of TXNIP under diabetic conditions, demonstrating the expression level of TXNIP and GS during retinal neuron damage. After the TXNIP damage function was identified, working on the relevant pathways of TXNIP and using the inhibition or siRNA of TXNIP will provide new mechanisms and therapeutic targets for DR.

Material and Methods

Cell culture

Muller cells were obtained from retinas of neonate mice, digested by trypsin, and then cultured in 6-well plates. The cells were subcultured in DMEM (1:2 ratio) at a density estimated to reach 80% confluence. Immunohistochemistry and electron microscopy were used for morphological identification of Muller cells. After 2-4 generations in culture, Muller cells were diluted to 1×10^6/l, and then were washed 3 times in D-hank's solution. Afterwards, cells were divided into 4 groups with different treatments: cells cultured with DMEM medium without glucose as the control group, cells cultured with DMEM containing 25 mmol/l glucose as the model group, cells in the model group treated with azaserine as the intervention group, and cells in the model group treated with the same volume of phosphate-buffered saline (PBS) as the intervention control group. All cells were incubated at 37°C for 24 h.

Immunofluorescence

Muller cells were cultured with or without high glucose and grown to 80% confluence, then fixed in PBS containing 4% cross-linking agent (paraformaldehyde, 4%) for 20 min. Then cellular contents were washed with 0.05% Triton X-100 for 30 min and with PBS for 15 min. Cells were then incubated overnight with different primary antibodies against GS. Then, cells were washed 3 times in PBS with 5-min time intervals. Afterwards, fluorescein isothiocyanate (FITC) was used for cell immuno-labelling, and then cells were morphologically observed using an epifluorescence microscope.

Western blot

Muller cells in each group were homogenized in lysis buffer and the homogenate was centrifuged at 12,000×g for 15 min at 4°C. Then protein concentrations were measured by bicinchoninic

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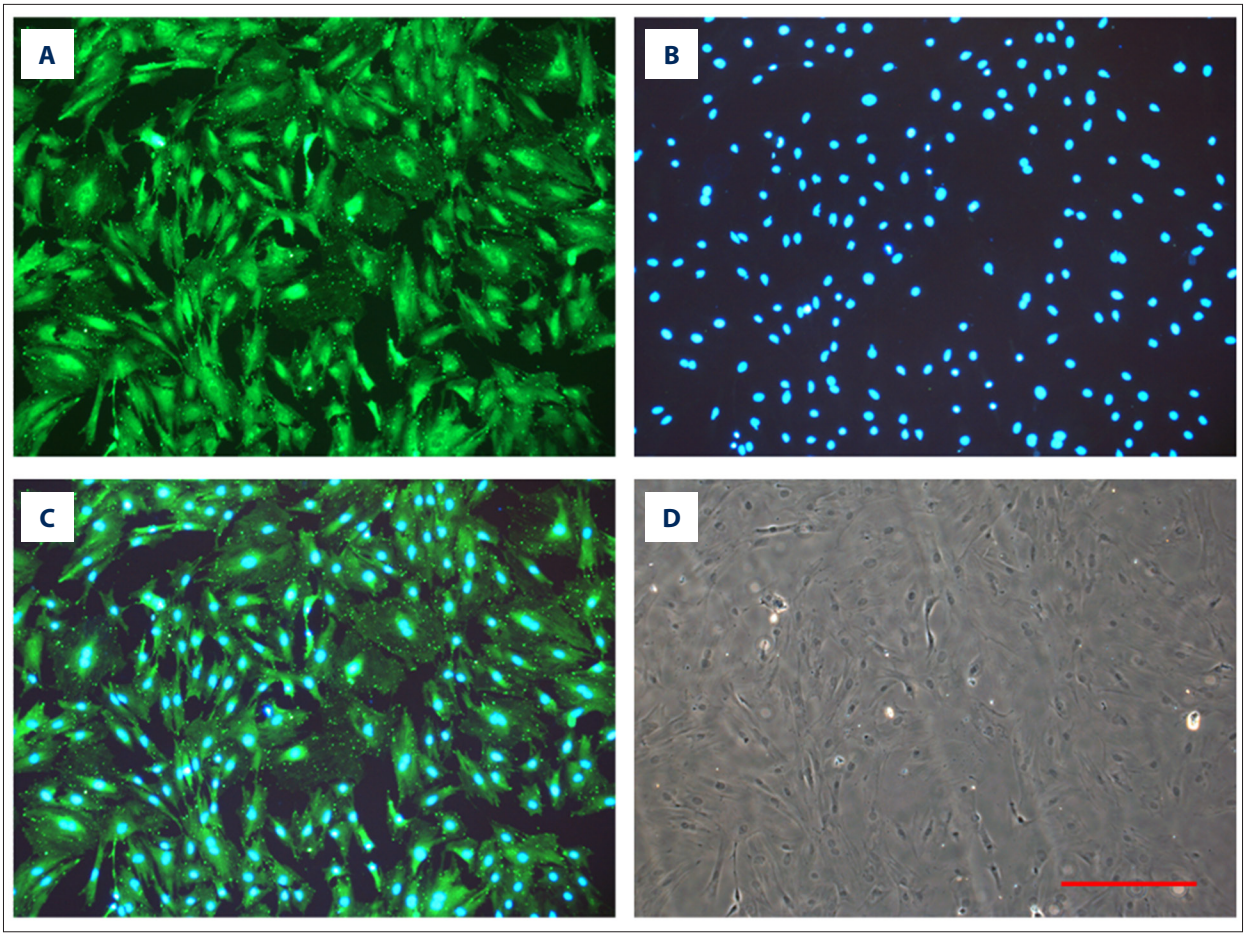

Figure 1. Identification of Muller cells by immunofluorescence. (**A**) GS expression in cells colored by FITC. (**B**) Cell nuclei dyed by DAPI. (**C**) Merged picture of (**A**) and (**B**). (**D**) Muller cells in white-light (scale bars, 100 μm).

acid technique. Proteins were resolved by polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Life Science, West Chester, PA, USA) for electrophoresis. The PVDF membranes were blocked at room temperature incubation for 2 h in TPBS containing 5% skim milk powder. They were then incubated with primary antibody (TXNIP 1:50) for 3 h at room temperature. Afterwards, the membranes were washed (3 times, 5 min every time) and incubated with the secondary antibody for 1 h at room temperature, followed by detection with Western blot reagent. To ensure that equal quantities were loaded in each lane, the membranes were blotted with anti-GAPDH antibody (Sigma Chemical Co., St Louis, MO, USA). BioRad Quantity One software was used to analyze absorbance value (A) of each band, including target protein and β-actin. The ratio of $A_{target\ protein}/A_{GAPDH}$ was considered as a relative integral A value presenting the expression level of each target protein.

Real time RT-PCR

The differently treated Muller cells were collected by centrifugation at 4°C, washed twice with chilled PBS (0.144% KH_2PO_4, 0.8% NaCl, 0.795% Na_2HPO_4), and then total RNA of differently treated cells was isolated, as previously described. Complementary DNA (cDNA) was synthesized using SuperScript II (Invitrogen Life Technologies, Baltimore, MD, USA) according to the manufacturer's protocol. Semi-quantitative analysis was carried out by use of the BioRad GS UV gel imaging system. The primer pairs used were: TXNIP-F: 5'-CGAGTCAAAGCCGTCAGGAT-3', TXNIP-R: 5'-TTCATAGCGCAAGTAGTCCAAGGT-3'; GS-F: 5'-GCCGTGGTGCTACTGATTGCT-3', GS-R: 5'-GCTTCCCAGTTCTGTGCGTTAT-3'; IL-1β-F: 5'-TGGCAGCTACCTATGTCTTGC-3', IL-1β-R: 5'-CCACTTGTTGGCTTATGTTCTG-3'. The PCR procedure was 36 cycles of 94°C for 1 min, 55–60°C for 1 min, and 72°C for 2 min, using the primers described above. The relative expression of the tested gene was defined as the copy number ratio of cDNA to that of GAPDH in the same sample.

Statistical analysis

All data analyses were performed with SPSS 19.0 software. Quantification of mRNA expression is presented as mean ± standard error, and data were submitted to Student's t-test

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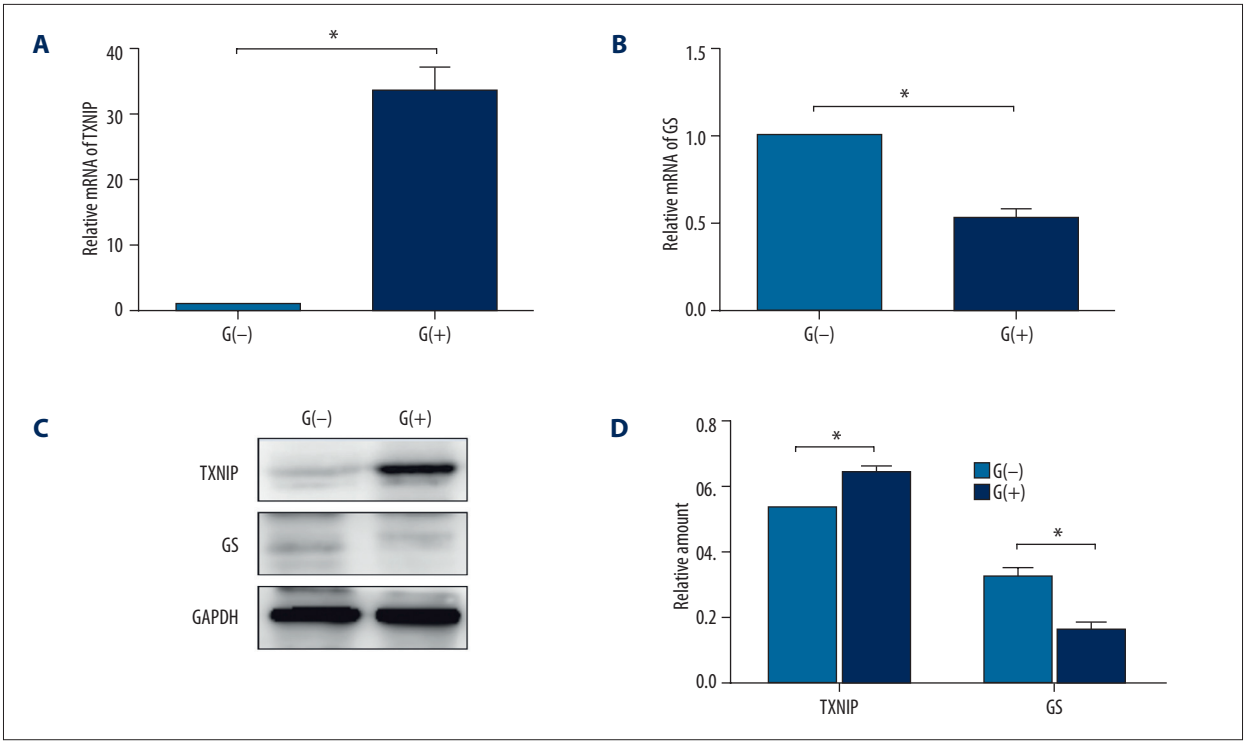

Figure 2. High glucose increased expression of TXNIP but showed inhibition effects on GS both at mRNA and protein levels. Relative mRNA expression levels of TXNIP (**A**) and GS (**B**) were analyzed by RT-qPCR and each measurement was performed in triplicate. (**C**) Expression levels of TXNIP and GS detected by Western blot. (**D**) Gray comparison of results in (**C**). * $P<0.05$.

to compare results between the 2 groups. All P values were 2-sided and a P value less than 0.05 was considered statistically significant.

Results

Culture and identification of Muller cells

GS was specifically expressed in Muller cells, which is a key enzyme that converts glutamate into glutamine; therefore, it can serve as a specific immune marker of Muller cells. In this study we selected GS to identify the separation of Muller cells (Figure 1) and the positive rate was about 90%.

High glucose affects expression levels of TXNIP and GS in Muller cells

Muller cells were cultured in DMEM containing high concentrations of glucose (25 mM) for 24 h. The protein and mRNA expression levels of TXNIP and GS in cells cultured with or without high glucose were detected by Western blotting and RT-qPCR, respectively. The results in Figure 2 show that a high-glucose condition increased TXNIP expressions at both protein and mRNA levels, but its effect on GS was the opposite.

Azaserine is responsible for TXNIP and GS expression in Muller cell

The expression of TXNIP is reduce by using azaserine to identify the expression of GS for further defining the possible mechanism of TXNIP in DR. To ascertain whether azaserine is involved in TXNIP and GS expression, we examined TXNIP and GS expression in Muller cells treated with or without azaserine. There was almost no TXNIP expressed in cells cultured without glucose, regardless of whether azaserine was added. However, when azaserine inhibited TXNIP expression in the high-glucose condition, the expression level was decreased by approximately 36±4% ($P<0.01$) (Figure 3A). Azaserine increased mRNA expression levels of GS cultured in high-glucose conditions (1.77±0.01-fold) or not (1.15±0.1-fold) (Figure 3B). Western blot analysis showed similar expression trends of TXNIP and GS protein when Muller cells were treated with azaserine (Figure 3C, 3D).

The role of IL-1β in retinal Muller cells

To confirm the reported results that IL-1β is associated with GS in retinal Muller cells at high glucose levels [11], we examined the mRNA and protein expression levels of IL-1β in Muller cells by using RT-PCR and Western blot. As shown in Figure 4, when cells were in normal conditions, azaserine has

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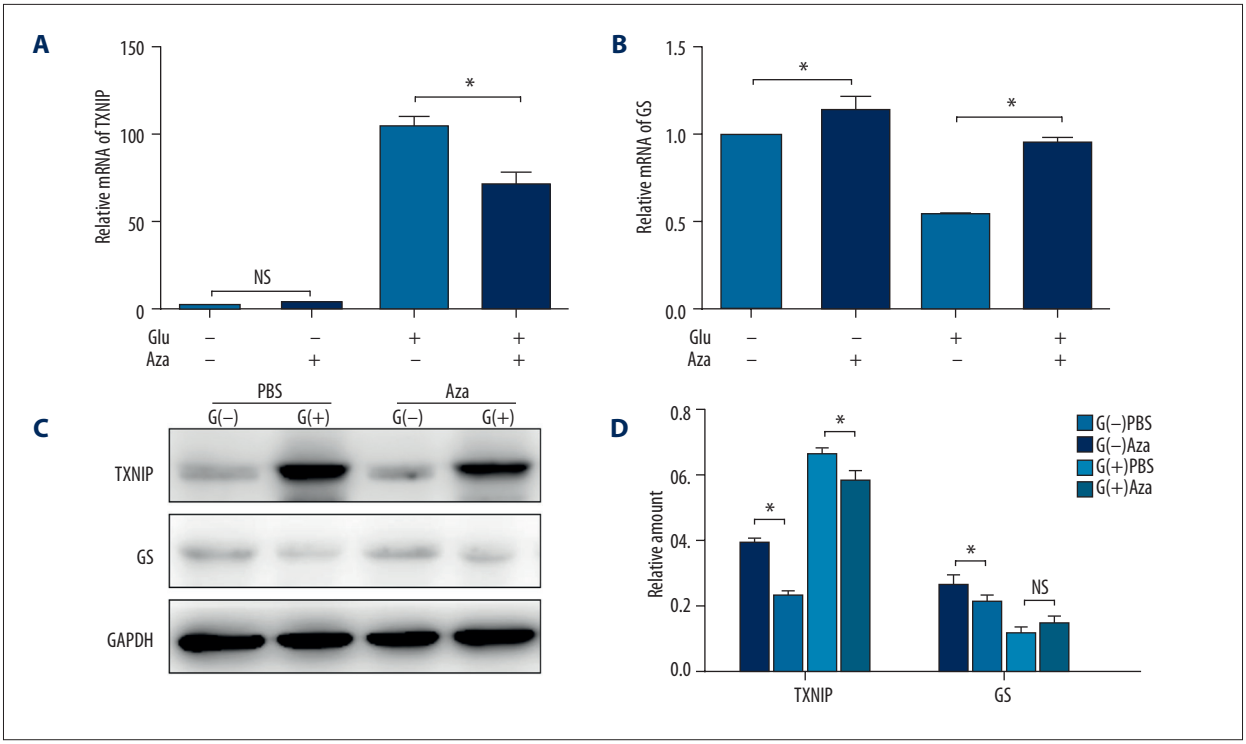

Figure 3. Azaserine affects TXNIP and GS expression in Muller cells. RT-qPCR analysis for TXNIP and GS mRNAs showed increased TXNIP expression (**A**) and decreased GS expression (**B**) induced by azaserine. (**C**) Western blot was used to detect protein expression levels of TXNIP and GS. (**D**) Gray comparison was carried out on the results in (**C**).

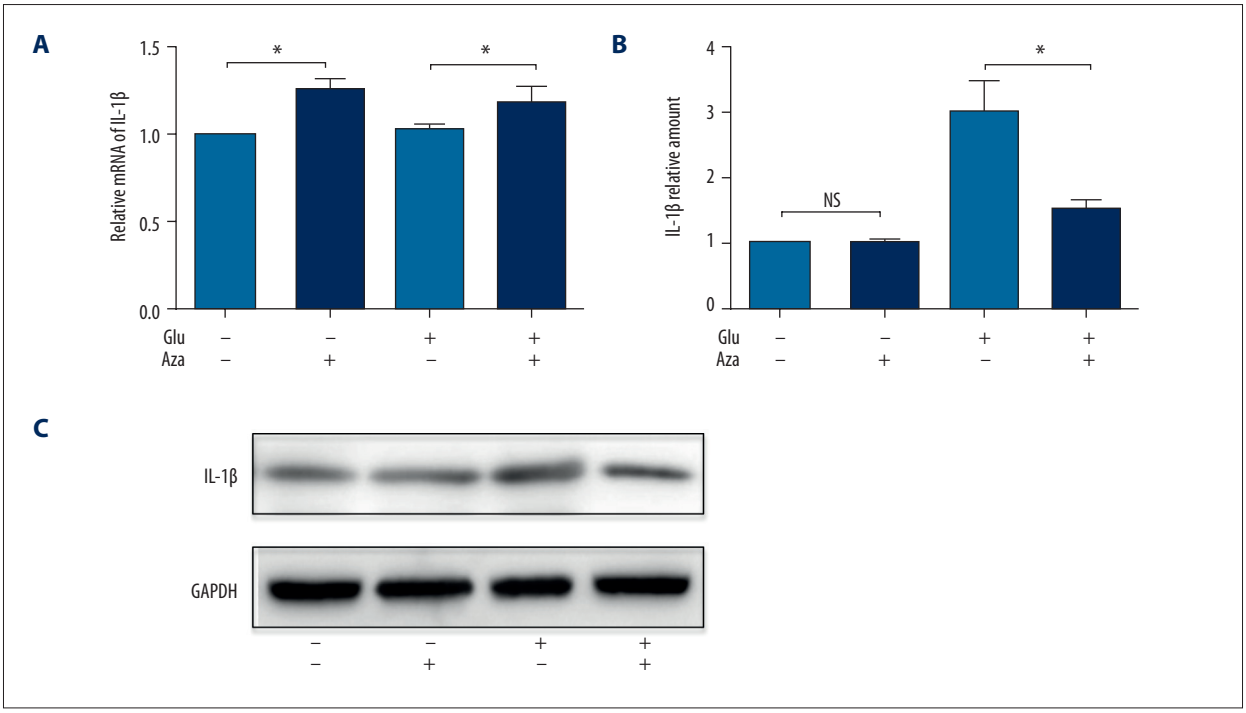

Figure 4. mRNA and protein expression levels of IL-1β in Muller cells cultured with or without 25 mM glucose. (**A**) Muller cells in different groups were cultured with or without 25 mM glucose medium for 24 h and mRNA expression levels of IL-1β were detected by RT-qPCR. After being treated with azaserine, Western blot analysis was done to detect protein expression of IL-1β (**C**) and gray comparison was carried out (**B**). Each measurement was performed in triplicate.

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no effect on mRNA and protein expression of IL-1β. However, when cells were cultured under high-glucose conditions, the expression of IL-1β at the protein level and mRNA level were decreased when azaserine was added.

Discussion

TXNIP works by binding protein to thioredoxin (TRX) protein. TRX, a small protein approximately 12 kD with oxidation-reduction activity, is widely distributed in prokaryotic and eukaryotic organisms. The TRX system consists of TRX, TRX reductase (TRXR or TR), and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The main biological function of TRX is to regulate the intracellular redox state, combat oxidative stress, and protect tissues or cells from damage. TXNIP [12], also known as vitamin D 3-regulated protein 1 (VDUP1) or TRX-binding protein 2 (TBP-2), weighs about 50 kD and has homology with inhibiting protein. TXNIP was initially found in leukemia cells (HL-60) treated with 1, 25-dihydroxyvitamin D3, and then it was separated by a yeast 2-hybrid system. TXNIP is considered a TRX-binding protein, which negatively regulates expression and function of TRX. Inhibiting the function of the TRX system plays a mediating role in oxidative stress, and has multiple roles in oxidation reduction, cell proliferation, apoptosis, and lipid and glucose metabolism.

A high-glucose environment was found to cause TXNIP overexpression and lead to excessive ROS production in the islet cells through activation of carbohydrate reaction element-binding protein (ChREBP) on the promoter of TXNIP [13]. Other studies suggested that high-glucose conditions increased expression of TXNIP via p38 mitogen-activated protein kinase (MAPK) and forkhead box transcription factor O subfamily 1 (FOX01) kinase pathways. It also leads to excessive ROS production in islet cells, and p38 MAPK can activate ROS, thereby creating a vicious cycle [14]. Hamada et al. [15] constructed an STZ-induced diabetic rat model and used enzyme-linked immunosorbent assay (ELISA) to detect the level of oxidative stress markers 8-OHdG and acrolein adduct in rat kidneys. They reported that both oxidative stress markers were significantly increased in the experimental group compared with the control group. The results of RT-qPCR demonstrated that mRNA level of TXNIP in rat kidneys in the experimental group were higher than in the control group, suggesting that a high level of TXNIP is a potential mechanism for maintaining high levels of oxidative stress in a diabetic internal environment.

In the field of ophthalmology, Takhellambam et al. [10] found that high concentrations of glutamate can upregulate the expression of TXNIP, indicating that TXNIP is also related to glutamate. However, the study did not further explore the relationships between TXNIP and glutamate and glutamine synthase, so it could not discover the exact pathological mechanism. In addition, Perrone et al. [16] found that azaserine inhibits the hexosamine biosynthesis pathway (HBP). HBP pathway activation increases the expression of TXNIP, which in turn increases the expression of Cox-2 and FN downstream. As a nervous system excitatory neurotransmitter, excessive glutamate can cause ganglion cell damage and death. Glutamate maintains its balance by glutamate transporters and GS in Muller cells, and dysfunction of either of them will lead to glutamate metabolic disorder.

Our previous studies [17–21] found that in the retina of diabetic rats, GS was generated from a few continuous filaments in the inner nuclear layer, and was predominantly expressed on the retinal ganglion cell layer and inner nuclear layer. GS expression was significantly decreased compared with the control group at 3 days to 1 month, although no significant difference was found in the first 2 months. In the condition of high glucose, accumulation of GS in Muller cells was decreased, resulting in the dysfunction of Muller cells, and further induced the apoptosis of ganglion cells. It was also reported that in high-glucose conditions, IL-1β remarkably up-regulated the level of c-Jun and reduced the level of GS, suggesting that IL-1β may play a role in the development of DR (11). The results of our study are consistent with previous research results, suggesting that in the early stage of DR, TXNIP suppresses the expression of GS by IL-1β.

Conclusions

The present study demonstrates that diabetes induces TXNIP expressions in mRNA levels, but showed the opposite effect on GS. IL-1β plays an important role in this pathway. Moreover, azaserine can effectively increase the expression of GS via attenuating the expression of TXNIP, which provides a possible treatment for DR and lays a new theoretical foundation for the clinical treatment of DR and other diabetic microvascular changes.

Conflict of interest

All authors declare no conflicts of interest.

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