Thioredoxin is implicated in the anti-apoptotic effects of grape seed proanthocyanidin extract during hyperglycemia

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Abstract. Diabetic retinopathy has long been recognized as a microvascular disease, however, recent research has indicated that diabetic retinopathy may also be considered a neurodegenerative disease. The elucidation of the molecular mechanisms underlying the development of diabetic retinopathy is imperative for the development of preventive and treatment strategies for patients with diabetes. In the present study, grape seed proanthocyanidin extract (GSPE) was used to upregulate the expression of thioredoxin (Trx), in order to evaluate its potential as a novel agent for the prevention and treatment of neurodegenerative diseases, including diabetic retinopathy. Hematoxylin and eosin staining was performed to observe the morphology of retinal neurons, whereas flow cytometry and terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5'-triphosphate nick-end labeling were employed to investigate cellular apoptosis. Reverse transcription-quantitative polymerase chain reaction and western blot analysis were performed to assess the mRNA and protein expression of target proteins in order to investigate the underlying molecular mechanisms. In vivo, it was found that the photoreceptor cell was damaged in diabetic mice but following GSPE treatment, the process could be inhibited. In vitro, the results of the current study demonstrated that, under hyperglycemic culture conditions, the expression of 78 kDa glucose-regulated protein, which is an endoplasmic reticulum stress marker, was upregulated. In addition, the expression of Trx was downregulated and cell apoptosis was enhanced. Notably, treatment with GSPE was revealed to inhibit the neurodegenerative process induced by hyperglycemia. However, treatment with the Trx inhibitor PX12 in combination with GSPE was demonstrated to potentiate apoptosis compared with GSPE treatment alone under hyperglycemic conditions. Furthermore, the protein expression of apoptosis signal-regulating kinase (ASK) 1 and Trx-interacting protein (Txnip) was also upregulated by hyperglycemia, whereas GSPE was revealed to counteract this upregulation. In conclusion, the results of the present study indicate that Trx may be implicated in the mechanisms underlying the protective effects of GSPE against hyperglycemia-induced cell degeneration and apoptosis. The molecular mechanisms may also involve inhibition of the activation of the Trx/ASK1/Txnip signaling pathway.

Introduction

Neurodegenerative diseases, including Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease and Huntington's disease, are predicted to emerge as the second leading cause of mortality worldwide by 2040. Therefore, understanding the molecular mechanisms underlying neurodegeneration, and developing effective therapeutic strategies is critical (1). Neurodegenerative diseases are also considered to be protein misfolding disorders, and they share similar characteristics, such as aggregation of misfolded proteins, which may lead to neuronal dysfunction and apoptosis. Previous research has indicated that the processing of misfolded aggregated proteins in the endoplasmic reticulum (ER) induces ER stress and causes mitochondrial dysfunction, and these processes are critical in the progression of neurodegenerative diseases (2).

Diabetic retinopathy is largely considered to be a microvascular disease, however, structural neurodegenerative alterations, such as neuronal apoptosis, have been described in the early stages of diabetic retinopathy (3,4). Therefore, diabetic retinopathy is recognized as a neurodegenerative disorder, as well as a microvascular disease. In patients with diabetes, a non-enzymatic reaction between sugars and the amino groups of proteins, lipids and nucleic acids leads to the reversible formation of Schiff bases. Following rearrangement, they form Amadori products, and these early glycation products then undergo irreversible cross-linking, forming heterogeneous fluorescent derivatives that are termed advanced glycation
end-products (AGEs) (5). AGEs are a type of misfolded protein that have the ability to induce ER stress, subsequently leading to mitochondrial dysfunction and oxidative stress (6).

Oxidative stress has been reported to be a principal mechanism implicated in the pathogenesis of various diseases, including neurodegenerative disorders, diabetes and cardiovascular disease (7). In addition, oxidative stress has been implicated in a number of pathologies associated with diabetes, including diabetic neuropathy and nephropathy (8). Therefore, antioxidative agents that prevent or delay oxidative stress-induced neuronal apoptosis may have potential in the development of effective therapeutic strategies to prevent the neurodegenerative processes associated with diabetes.

Thioredoxin (Trx) was initially discovered in 1964 and belongs to the Trx family of redox proteins. It is a 12 kDa multifunctional protein with a redox-active disulfide/dithiol within its conserved active site sequence (-Cys-Gly-Pro-Cys-) (9). Trx functions in several cellular responses, including proliferation, apoptosis and survival. In the extracellular milieu, Trx exhibits chemokine-like activity, and functions as a scavenger of reactive oxygen species and transcription factor activator in the cytoplasm (10). Trx has also been reported to serve a cytoprotective role under conditions of cellular stress and injury (11).

Proanthocyanidins are phenolic compounds that are presented in fruit, vegetables, wine, tea, nuts and seeds (12). Grape seed proanthocyanidin extract (GSPE) consists of a mixture of pycnogenols and flavonoids, including oligomeric proanthocyanidins, which exhibit potent antioxidative properties and are extracted from grape seeds and skins (13). A previous study reported that GSPE suppressed the apoptosis of bladder cells in diabetic rats by activating the nuclear factor erythroid 2-like 2 (Nrf2) pathway (13). Furthermore, a previous study by our group demonstrated that activation of the Nrf2 pathway led to upregulation of Trx in mice with photoreceptor degeneration (14). Therefore, in the present study, GSPE was used to upregulate the expression of Trx, and to investigate the antiapoptotic effects of Trx in hyperglycemia-induced neurodegeneration. The present study aimed to provide evidence to support the potential use of GSPE for the prevention and treatment of diabetic retinopathy in clinical practice.

Materials and methods

Cell culture and reagents. Mouse Neuro2a neuroblastoma cells obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a 5% CO₂ atmosphere. Then, 3x10⁵ cells/ml were seeded into a 6-well plate and medium was replaced every 1-2 days. The cells were washed with PBS prior to the experiments. The Trx inhibitor PX12 (Tocris Bioscience; Bio-Technne, Minneapolis, MN, USA) was dissolved in 1 mM dimethyl sulfoxide and stored at -20°C. The stock high-glucose (HG; 100 mM) medium (Gibco; Thermo Fisher Scientific, Inc.) was diluted with DMEM to give a final working concentration of 30 mM glucose and was stored at 4°C. GSPE was purchased from Tianjin Jianfeng Natural Product R&D Co., Ltd. (Tianjin, China). Neuro2a cells were pretreated for 6 h with or without 10 µM PX12, and subsequently received treatment for 24 h with or without 10 µg/ml GSPE and 30 mM glucose at 37°C in a 5% CO₂ atmosphere.

Flow cytometric analysis. Neuro2a cells that were treated with HG, HG + GSPE or HG + GSPE + PX12, and control cells, were washed with PBS three times prior to analysis. Then, 3x10⁶ cells/ml were collected by centrifugation at 112 x g for 5 min at room temperature, and then stained with annexin V (1:100)propidium iodide (PI) (1:500) in binding buffer (BioTools, Inc., Jupiter, FL, USA) at room temperature for 15 min in the dark. Subsequently, the samples were analyzed by BD Accuri™ C6 software version 1.0.264.21 (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. Total proteins were extracted from Neuro2a cells that were treated with HG, HG + GSPE or HG + GSPE + PX12, and control cells. The cells were lysed in ice-cold lysis buffer (Geno Technology, Inc., St., Louis, MO, USA) for 30 min. The lysates were centrifuged at 12,000 x g for 30 min at 4°C, and the supernatants were collected as protein samples. The concentration of protein was measured by BCA assay and equal amounts of extracted protein samples (50 µg protein/lane) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat milk for 1 h at room temperature and then incubated with the following primary antibodies overnight at 4°C:

- Anti-Trx (rabbit; 2429; 1:1,000 Cell Signaling Technology, Inc., Danvers, MA, USA), anti-78 kDa glucose-regulated protein (rabbit; GRP78; 11587-1-AP; 1:1,000); ProteinTech Group, Inc., Chicago, IL, USA), anti-Trx-interacting protein (TNnip; rabbit; 18243-1-AP; 1:1,000); ProteinTech Group, Inc.), anti-apoptosis signal-regulating kinase (ASK) 1 (rabbit; ab45178; 1:2,000; Abcam, Cambridge, UK), anti-Nrf2 (rabbit; sc-722; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-β-actin (mouse; sc-4778; 1:1,000; Santa Cruz Biotechnology, Inc.). All antibodies were diluted in 5% non-fat milk. The membranes were washed three times with 1X TBS containing 0.1% Tween-20 (TBST) for 15 min and then incubated with secondary antibodies horseradish peroxidase (HRP) conjugated goat anti-rabbit immunoglobulin (Ig) G (ab6721; 1:2,000; Abcam) and HRP goat anti-mouse IgG (ab205719; 1:2,000; Abcam) for 1 h at room temperature. Following three washes with 1X TBST for 20 min, the protein bands were visualized using enhanced chemiluminescence (GE RPN2232; GE Healthcare Life Sciences) and exposed to X-ray film. Blots were semi-quantified by densitometry using Labworks software version 4.5 (Perkin Elmer, Inc., Waltham, MA, USA).

Terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphosphate nick-end labeling (TUNEL) assay. To detect cell apoptosis, 3x10⁵ cells/ml was seeding into 8-well chamber slides (Thermo Fisher Scientific, Inc.) and a TUNEL assay was performed using a FITC labeled TUNEL Cell Death Detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), according to the manufacturer's protocol. Briefly, following control, HG, HG + GSPE or HG + GSPE + PX12 treatment, cells were fixed in 4% paraformaldehyde for 15 min at room temperature.
temperature, washed in PBS three times for 10 min and incubated with 50 µl reaction mixture for 60 min at 37°C in a dark humidified chamber. The cells were then counterstained with PI (Vector Laboratories, Inc., Burlingame, CA, USA) diluted 1:500 for 10 min at room temperature in the dark. Apoptotic cells were observed in at least 5 fields under a fluorescence microscope (BZ 7000; Keyence Corporation, Osaka, Japan) and Image Pro Plus version 5.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to analyze data.

Animal experiments. A total 24 male inbred BALB/c mice (age, 6 weeks; weight, 20-25 g) were supplied by Dalian Medical University (Liaoning, China) and were housed in an animal control facility for 2 weeks in a 12-h light/dark cycle with a mean illumination of 80 lx and 30-70% humidity. The animals were maintained at 22±2°C. Tap water and food pellets (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) were available ad libitum. All experimental procedures were conducted in accordance with the institutional guidelines for the care and use of laboratory animals, and experimental protocols were approved by the Institutional Animal Care and Use Committee at Dalian Medical University. A total of 6 mice were assigned to 4 groups; control, diabetic, diabetic + GSPEI and diabetic + GSPEII. The diabetic mice were fed a high fat diet (10% lard, 20% yolk, 1% cholesterol, 0.5% cholate, 20% sucrose and 48.5% standard diet) for 8 weeks and intraperitoneally injected with streptozotocin (STZ; 60 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) twice every 3 days to induce diabetes. When blood glucose levels reached 250 mg/dl, the model was considered to be successfully established. Subsequent experiments were conducted between 10.00 am and 2.00 pm. STZ was dissolved in cold 50 mM citric acid buffer (pH 4.5). The diabetic mice were also administered with various concentrations of GSPE (50 and 100 mg/kg) via oral gavage.

Morphological analysis by quantitative histology. The animals were sacrificed with an overdose of carbon dioxide 2 weeks after commencement of the different treatments. The enucleated eyes were immersed in Bouin’s solution for 24 h and then fixed in 70% ethanol for 24 h at room temperature. Following alcohol dehydration, the eyes were embedded in paraffin and 5-µm sagittal sections containing the entire retina, including the optic disc, were obtained. The retinal sections were stained with 0.5% hematoxylin and 1% eosin (H&E) at room temperature to observe under a light microscope. In each of the superior and inferior hemispheres, the thickness of the outer nuclear layer (ONL) was measured at 9 defined points. Each point was centered on adjacent 220-µm lengths of the retina. The first point of measurement was at a ~220 µm distance from the optic nerve head, and subsequent measurement points were located in the periphery. The average ONL thickness was determined based on measurements from 18 points in each section.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was obtained from each retina sample using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). RT was performed with the PrimeScript RT Reagent kit (Perfect Real Time; Takara Bio, Inc., Otsu, Japan). RT-qPCR was performed to measure Trx mRNA expression using SYBR Premix DimerEraser (Takara Bio, Inc.), with reverse-transcribed cDNA as the template. All PCR reactions were conducted in a final volume of 20 µl. The amplification was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: 95°C for 30 sec followed by 40 cycles of 95°C for 3 sec, 72°C for 30 sec and 55°C for 30 sec. GAPDH was used as an internal reference gene. The primers used were as follows: Forward, 5'-GGAATTTGAAGACGATCGAG-3' and reverse, 5'-ACGCCCTAGAATCTCATTAAT-3' for Trx; forward, 5'-TGTGATGGTGTGAAACCATCGAA-3' and reverse, 5'-GACCCCCTTCCACAATGGCCAAAGTTT-3' for GAPDH. The 2^(-ΔΔCt) method was used to quantify the results (15).

Statistical analysis. Data are presented as the mean ± standard deviation. Each experiment was performed three times. The statistical significance of the differences between groups was assessed using one-way analysis of variance. Statistical analysis was performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Antiprotective effects of GSPE during in vivo hyperglycemia. In the present study, diabetes was induced in mice in vivo using an STZ injection and a high-fat diet. The morphology of the retina was observed following H&E staining and the present results demonstrated that ONL (photoreceptor cell layer) thickness was diminished in diabetic mice compared with in healthy mice. However, following treatment with various concentrations of GSPE, ONL degeneration was revealed to be prevented (Fig. 1A and B). Reverse transcription-quantitative polymerase chain reaction was used to detect the expression of Trx in tissue samples isolated from mice in the various groups. As presented in Fig. 1C, the mRNA expression of Trx was significantly downregulated in diabetic mice compared with in healthy mice. Conversely, following GSPE administration, Trx mRNA expression levels were significantly increased compared with in diabetic mice.

Antiprotective effects of GSPE during in vitro hyperglycemia. During the in vitro experiments used in the present study, mouse Neuro2a cells were maintained in hyperglycemic conditions (30 mM glucose) to induce an HG damage model with or without GSPE treatment. Hyperglycemia-induced apoptosis in Neuro2a cells was quantitatively analyzed using flow cytometry. As presented in Fig. 2A and B, HG treatment increased the percentage of apoptotic cells compared with the control group, whereas treatment with GSPE counteracted the proapoptotic effects of hyperglycemia. Western blot analysis was used to detect the expression of GRP78, which is one of the markers of ER stress, Nrf2 and Trx in Neuro2a cells. The present results demonstrated that the protein expression of GRP78 was significantly upregulated in HG-exposed cells, whereas the protein expression of Nrf2 and Trx was significantly downregulated (Fig. 2C-F).
However, following GSPE treatment, GRP78 expression was significantly decreased, whereas Nrf2 and Trx expression was significantly increased (Fig. 2C-F). These results indicate that HG treatment may induce ER stress in neuronal cells and downregulate the expression of Trx, thus leading to cell apoptosis; Trx may therefore be implicated in the antiapoptotic effects observed following GSPE treatment.

Roles of Trx during hyperglycemia-induced Neuro2a cell apoptosis. Neuro2a cells were pretreated for 6 h with or without the Trx system inhibitor PX12 (10 µM), following which they were treated for 24 h with or without 10 µg/ml GSPE and 30 mM glucose. Neuro2a cell apoptosis was analyzed using TUNEL staining (Fig. 3A and B) and flow cytometry (Fig. 3C and D). The present results demonstrated that GSPE...
Figure 3. Effects of Trx inhibition on the apoptosis of mouse Neuro2a cells induced by hyperglycemia in vitro. (A) TUNEL staining was used to assess the effects of Trx inhibition by PX12 on hyperglycemia-induced apoptosis. Nuclei were stained with DAPI. Scale bar, 50 µm. (B) TUNEL staining results were quantified and statistically analyzed. (C) Flow cytometry was used to assess the effects of Trx inhibition by PX12 on hyperglycemia-induced apoptosis. (D) Apoptosis percentages obtained by flow cytometry were statistically analyzed. Data are expressed as the mean ± standard deviation (n=3 for each group). *P<0.05, **P<0.01 and ***P<0.001, as indicated. Trx, thioredoxin; TUNEL, terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphate nick-end labeling; HG, high glucose; GSPE, grape seed proanthocyanidin extract; PI, propidium iodide.

Figure 4. Inhibition of Trx by PX12 alters the expression of molecules implicated in the Trx cell signaling pathway under hyperglycemic conditions. Western blot analysis was used to detect the expression of Trx, Txnip and ASK1 in Neuro2a cells. (A) Representative blot demonstrating expression of Trx, Txnip and ASK1. β-actin was used as the loading control. The protein expression levels of (B) Trx, (C) Txnip and (D) ASK1 were assessed using densitometric analysis. Data are expressed as the mean ± standard deviation (n=3 for each group). *P<0.05, **P<0.01 and ***P<0.001, as indicated. Trx, thioredoxin; Txnip, Trx-interacting protein; ASK, apoptosis signal-regulating kinase; HG, high glucose; GSPE, grape seed proanthocyanidin extract.
inhibited Neuro2a apoptosis induced by hyperglycemia. Conversely, PX12 treatment was revealed to significantly enhance the HG-induced Neuro2a cell apoptosis compared with GSPE-treated cells (Fig. 3) and inhibit the expression of Trx (Fig. 4). These results indicate that, under hyperglycemic conditions, GSPE may exhibit neuroprotective effects in Neuro2a cells that may depend on a Trx-mediated mechanism.

Effects of Trx expression inhibition in HG-induced Neuro2a cell apoptosis. In order to investigate the molecular mechanisms involved in Trx-mediated neuroprotection, Neuro2a cells were treated with HG, GSPE and PX12, and the protein expression levels of ASK1, Txnip and Trx were assessed using western blot analysis. The present results demonstrated that the expression of ASK1 and Txnip was upregulated in HG-treated cells, whereas the expression of Trx was downregulated compared with control cells (Fig. 4). Compared with the HG group, GSPE treatment significantly increased the protein expression levels of Trx, and decreased the levels of ASK1 and Txnip (Fig. 4). However, in cells pretreated with PX12, the expression of ASK1 and Txnip was increased, whereas the expression of Trx was decreased compared with GSPE-treated cells (Fig. 4). These results indicate that Trx-associated pathways may be implicated in the antiapoptotic effects of GSPE in hyperglycemic Neuro2a cells.

Discussion

Acute and chronic neurodegenerative diseases, including stroke, traumatic brain injury, and Alzheimer's and Parkinson's disease, are associated with high morbidity and mortality, and few effective therapeutic strategies are available for the treatment of these diseases (16). Previous studies have indicated that diabetic retinopathy may represent a novel type of neurodegenerative disease (4,17,18).

Diabetic retinopathy is the most common cause of irreversibly blind adults and leads to loss of central vision. It has long been considered a microvasculopathy, however, retinal diabetic neuropathy may also occur in patients with diabetes mellitus (19). Several events may lead to neuronal apoptosis in diabetic retinopathy, including mitochondrial dysfunction and ER stress. Previous studies have reported that ER stress is implicated in numerous diseases, including diabetes, neurodegeneration and cancer (20-22). In the present study, the protein expression of GRP78, which is an ER stress marker, was revealed to be upregulated following HG treatment in Neuro2a cells in vitro. These results indicate that ER stress-associated mechanisms were activated during hyperglycemia. In addition, Nrf2 protein expression was downregulated, which led to a decrease in Trx protein expression, subsequently leading to cell apoptosis. The present results indicate that Trx may have potential as a novel therapeutic target for the inhibition of HG-induced apoptosis.

GSPE is extracted from grape seeds and skins, and has been demonstrated to exert protective effects against various diseases, including diabetic nephropathy, drug-induced renal toxicity, cancer metastasis and ischemic cardiomyopathy in animal models (23). In addition, GSPE has been reported to activate the Nrf2 pathway, and Nrf2 pathway activation has been reported to be associated with the upregulation of Trx expression (13,14), thus indicating that Trx may be implicated in the antiapoptotic effects of GSPE. In the present study, GSPE was used to treat diabetic mice in vivo, and morphological analysis revealed that it was able to prevent ONL degeneration compared with untreated diabetic mice. In addition, the expression of Trx was significantly upregulated in mouse tissue samples following GSPE treatment compared with untreated diabetic mice.

Based on the aforementioned findings, the Trx inhibitor PX12 was used to pretreat Neuro2a cells. Flow cytometry and TUNEL staining demonstrated that the percentage of apoptotic cells was significantly reduced following GSPE treatment under hyperglycemic conditions. However, PX12 pretreatment inhibited the protective effects associated with GSPE treatment in vitro.

In the present study, the molecular mechanisms underlying the effects of GSPE were also investigated in vitro. Txnip is an endogenous Trx inhibitor that inhibits the intracellular actions of Trx, and Txnip has been reported to be upregulated in a hyperglycemic environment (24). ASK1 is a member of the mitogen-activated protein kinase kinase family that serves a major role during stress-induced apoptosis (25) and is regulated by Trx. In the present study, the protein expression levels of ASK1 and Txnip were increased following HG exposure, whereas it was decreased following GSPE treatment compared with untreated cells exposed to HG. However, ASK1 and Txnip expression was upregulated in GSPE-treated cells that were pretreated with PX12 compared with cells treated with GSPE alone. These results indicate that hyperglycemia may induce the apoptosis of Neuro2a cells by activating ER stress, thus leading to the activation of the Trx/ASK1/Txnip pathway. Based on these results, it may be hypothesized that Trx serves a key role in the antiapoptotic actions of GSPE.

In conclusion, the results of the present study demonstrated that hyperglycemia induced Neuro2a cell apoptosis via activation of ER stress-associated mechanisms and the downregulation of Trx expression. In addition, Trx may serve an important role in GSPE-mediated antiapoptotic mechanisms, thus implicating the Trx/ASK1/Txnip signaling pathway in HG-induced neurodegeneration. It can provide some evidence for potential treatment of patients with diabetic retinopathy.

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