Protective function of pyridoxamine on retinal photoreceptor cells via activation of the p-Erk1/2/Nrf2/Trx/ASK1 signalling pathway in diabetic mice

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Abstract. The present study aimed to investigate the mechanisms that mediate the protective effects of pyridoxamine (PM) on light-damaged retinal photoreceptor cells in diabetic mice. A high-fat diet and streptozotocin were used to induce a mouse model of type II diabetes. During the experiment, mice were divided the mice into three types of group, as follows: Control groups (negative control and light-damaged groups); experimental groups (diabetic and diabetic light-damaged groups); and treatment groups (25, 50 and 100 mg/kg PM-treated groups). Using hematoxylin-eosin staining, the number of nuclear layer cells were counted. Western blotting and immunohistochemistry were performed to measure the levels of thioredoxin (Trx), phospho-extracellular signal-regulated kinase 1/2 (p-Erk1/2), nuclear factor erythroid 2-related factor 2 (Nrf2) and apoptosis signal-regulating kinase 1 (ASK1). The photoreceptor cell count in the outer nuclear layer of the light-damaged, diabetic control and diabetic light-damaged groups were significantly reduced compared with the negative control group (P<0.001). The cell counts in the PM-treated groups were significantly increased compared with the diabetic group (P<0.001). Compared with the negative control group, the light-damaged, diabetic and diabetic light-damaged groups exhibited significantly decreased Trx, p-Erk1/2 and Nrf2 expression levels (P<0.001), and significantly increased ASK1 expression levels (P<0.001). However, in the PM-treated groups, Trx, p-Erk1/2 and Nrf2 expression levels were significantly increased (P<0.001), and ASK1 expression was significantly decreased (P<0.001). The results of the present study demonstrate that PM protects retinal photoreceptor cells against light damage in diabetic mice, and that its mechanism may be associated with the upregulation of Trx, p-Erk1/2 and Nrf2 expression, and the downregulation of ASK1 expression.

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Introduction

Diabetic retinopathy (DR) is the most common chronic microvascular complication of diabetes mellitus (DM) and is a leading cause of visual loss among working-age individuals. DR is an ocular manifestation of DM, affecting ≤80% of all patients that have had DM for ≥10 years (1). The normal functions of the retinal vasculature, neurons and resident glial cells are affected by DR. Several factors, including hyperglycaemia, advanced glycation end-products (AGEs) and cytokines, including vascular endothelial growth factor, have been implicated in the disease pathogenesis (2). Although the mechanism of DR has not been fully elucidated, it is understood that the oxidative damage induced by these factors contributes to the development of DM and DR (3). In summary, decreasing oxidative damage may be a therapeutic strategy for DR.

Thioredoxin (Trx) is a 12-kDa protein with a redox-active dithiol in its active site, -Cys-Gly-Pro-Cys-. It is a ubiquitous antioxidant enzyme with an essential role in various cellular functions. Trx was first identified in *Escherichia coli* in 1964 by Laurent *et al* (4). Trx has different forms depending on its cellular environment and is a major antioxidant important for the maintenance of redox balance within the cell (5). Furthermore, Trx is important in anti-apoptotic signalling and regulates the expression of certain transcription factors. It is also a critical regulator of apoptosis signal-regulating kinase 1 (ASK1) function.

Pyridoxamine (PM), a vitamin B6 metabolite, has been demonstrated to be a potent inhibitor of AGE formation *in vitro* and in animal models (6). PM was previously demonstrated to inhibit several oxidative and glycoxidative pathways that can cause protein damage (7). Additionally, PM is a prospective

drug for the treatment of diabetic nephropathy (8). However, little has been reported regarding the effects of PM on the retina.

Based on previous studies, the present study treated type II diabetic (T2D) mice exposed to light damage with PM to investigate the protective effects and mechanism of action of PM on light-induced retinal photoreceptor cell damage in diabetic mice.

Materials and methods

Animals. All experimental procedures were conducted in accordance with institutional guidelines for the Care and Use of Laboratory Animals, and protocols were approved by the Institutional Animal Care and Use Committees of Dalian Medical University Laboratory Animal Center (Dalian, China). A total of 42 male inbred BALB/c mice (Dalian Medical University Laboratory Animal Center) weighing ~35-45 g, aged 6 weeks, were housed in an animal colony facility for 2 weeks, with 6 mice per cage in each group. The animals were maintained in a room with a constant temperature (22±2˚C). All animals were born and raised in a 12-h on/off cyclic light environment at average (mean) illumination of 80 lx. Tap water and food pellets (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) were available *ad libitum*. After normal feeding for 4 weeks, mice in the T2D group (n=6) were injected intraperitoneally with 80 mg/kg streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA) dissolved in cold 50 mM citric acid buffer (pH 4.5; Beijing Solarbio Science & Technology Co., Ltd.) and fed a high fat diet (10% lard, 20% yolk, 1% cholesterol, 0.5% cholate, 20% sucrose and 48.5% standard diet) to induce T2D. When the fasting blood glucose (FBG; measured using a glucometer; Sinocare, Inc., Changsha, China) levels of the mice reached 11.1 mmol/l, the model was considered to be successfully established. Experiments were conducted between 10:00 a.m. and 2:00 p.m. PM (Sigma-Aldrich) was dissolved in normal saline and injected intraperitoneally at concentrations of 25 mg/kg (PMI group), 50 mg/kg (PMII group) and 100 mg/kg (PMIII group). The light damage was performed at 5,000 lux for 72 h. Animals were sacrificed by CO_2 asphyxiation after treatment.

HOMA-IR of serum insulin. Fasting serum insulin (FIN) levels were detected using an enzyme-linked immunosorbant assay kit (cat. no. BP-E20353; Shanghai Lengton Bioscience Co., Ltd., Shanghai, China). The insulin resistance index was determined based on the following equation: HOMA-IR = (FBG x FIN) / 22.5.

Morphological analysis by quantitative histology. The enucleated eyes of each mouse were immersed in 4% paraformaldehyde, containing 20% isopropanol, 2% trichloroacetic acid and 2% zinc chloride, for 24 h and then in 70% ethanol for 24-60 h. Following alcohol dehydration, the eyes were embedded in paraffin and 5 μm-thick sagittal sections containing the entire retina, including the optic disc, were sliced. The retinal sections were stained with hematoxylin-eosin (Beijing Solarbio Science & Technology Co., Ltd.). In each of the superior and inferior hemispheres, the outer nuclear layer (ONL) thickness was measured at nine defined points. Each point was centered on adjacent 220-μm lengths of the retina. The first point of measurement was ~220 μm from the optic nerve head, and subsequent measurement points were located more peripherally. The mean ONL thickness was determined based on 18 measurement points in each section.

Western blotting. Western blot analysis was performed as previously described (9). Briefly, retinas were lysed in radioimunoprecipitation assay buffer, and protein was quantified using a bicinchoninic acid kit (both purchased from Beyotime Institute of Biotechnology, Haimen, China). Then, protein was loaded (30 μg protein/lane), separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature and were then incubated overnight at 4˚C with the following primary antibodies: Monoclonal rabbit phospho-extracellular signal-regulated kinase 1/2 (p-Erk1/2; 1:1,000 dilution; cat. no. 4376; Cell Signaling Technology, Inc., Danvers, MA, USA); polyclonal mouse nuclear factor erythroid 2-related factor 2 (Nrf2; 1:1,000 dilution; cat. no. sc-722; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); and monoclonal β-actin (1:1,000 dilution; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). The membranes were washed three times with 1X Tris-buffered saline-0.1% Tween 20 (TBS-T; Beijing Solarbio Science & Technology Co., Ltd.) for 10 min. Subsequently, the membranes were incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated IgG for 1 h at room temperature and washed 3 times with 1X TBS-T for 15 min. The membranes were then developed using an enhanced chemiluminescence system and exposed to X-ray film (both purchased from Beijing Solarbio Science & Technology Co., Ltd.). The intensities of the bands were measured using LabWorks 4.5 software (Perkin Elmer, Waltham, MA, USA). All primary and secondary antibodies were diluted in TBS-T with 2.5% non-fat dry milk.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was obtained from each retina sample using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). 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'-GGAATGGTGAAGCAGATCGAG-3' and reverse, 5'-ACG CTTAGACTAATTCATTAAT-3' for Trx; forward, 5'-TGT GATGGGTGTGAACCACGAGAA-3' and reverse, 5'-GAG CCCTTCCACAATGCCAAAGTT-3' for GAPDH. The $2^{-\Delta\Delta Cq}$ method was used to quantify the results (10).

Immunohistochemical analysis. Paraffin-embedded retinal sections (5 μm) were deparaffinised, rehydrated and subjected

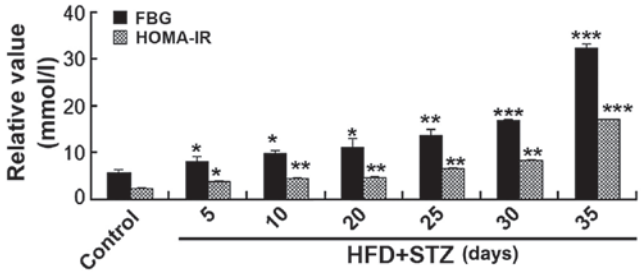

Figure 1. FBG and HOMA-IR levels in mice to determine establishment of a type II diabetic mouse model. Data are presented as the mean ± standard error of the mean, repeats = 6. *P<0.05, **P<0.01, ***P<0.001 vs. control. FBG, fasting blood glucose; HOMA-IR, homeostatic model assessment-insulin resistance; HFD, high fat diet; STZ streptozotocin.

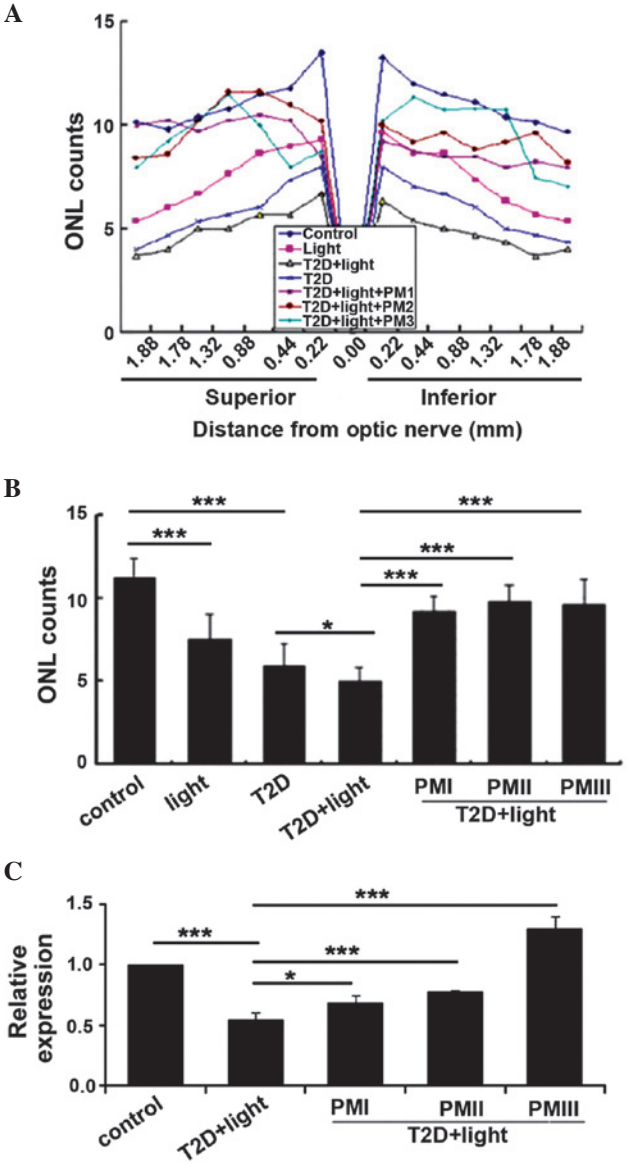

Figure 2. PM protects the retinal photoreceptor cells of T2D mice. (A) Photoreceptor ONL thickness measurements of each group (n=6). (B) Mean photoreceptor ONL thickness values of all measurements of the same group. (C) Thioredoxin (Trx) mRNA expression levels in the retinas of T2D mice treated with or without PM. Data are presented as the mean ± standard error of the mean, repeats = 3. *P<0.05, ***P<0.001. ONL, outer nuclear layer; T2D, type 2 diabetic; PM, pyridoxamine; PMI, 25 mg/kg PM; PMII, 50 mg/kg PM; PMIII, 100 mg/kg PM.

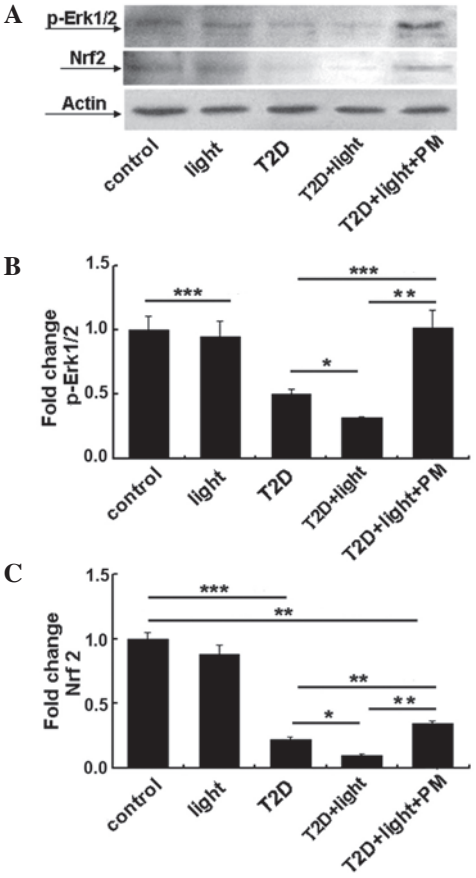

Figure 3. PM increases the expression of antioxidant-associated proteins in the retinas of T2D mice. (A) Western blotting and (B and C) densitometry analysis demonstrated that PM treatment (50 μg/kg) increased the protein levels of (B) p-Erk1/2 and (C) Nrf2 in the retinas of T2D mice. Data are presented as the mean ± standard error of the mean, repeats = 3. *P<0.05, **P<0.01, ***P<0.001. p-Erk1/2, phopsho-extracellular signal-regulated kinase; Nrf2, nuclear factor erythroid 2-related factor 2; T2D, type 2 diabetic; PM, pyridoxamine.

to antigen retrieval by boiling the sections in 10 mmol/l citrate buffer (pH 6.0; Beyotime Institute of Biotechnology) for 20 min and washing twice with water and twice with phosphate buffered saline (PBS). Sections were then blocked with bovine serum albumin (Beyotime Institute of Biotechnology) for 20 min and incubated with primary anti-ASK1 rabbit monoclonal antibody (dilution 1:50; cat. no. ab45178; Abcam, Cambridge, UK) at 4˚C overnight. For the analysis of ASK1 expression, after rinsing twice with PBS for 5 min, the sections were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin (dilution, 1:200; cat. no. ZB-2031; ZSGB-BIO, Beijing, China) at room temperature for 1 h. The sections were finally rinsed twice for 5 min in PBS and stained with 3,3'-diaminobenzidine (ZSGB-Bio). Image Pro Plus version 5.0 (Olympus Corporation, Tokyo, Japan) was used to analyze the integrated optical density index for each group.

Statistical analysis. Data are presented as the mean ± standard error of the mean. The statistical analyses were performed using one-way analysis of variance for continuous variables. SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA) was used for all of the statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

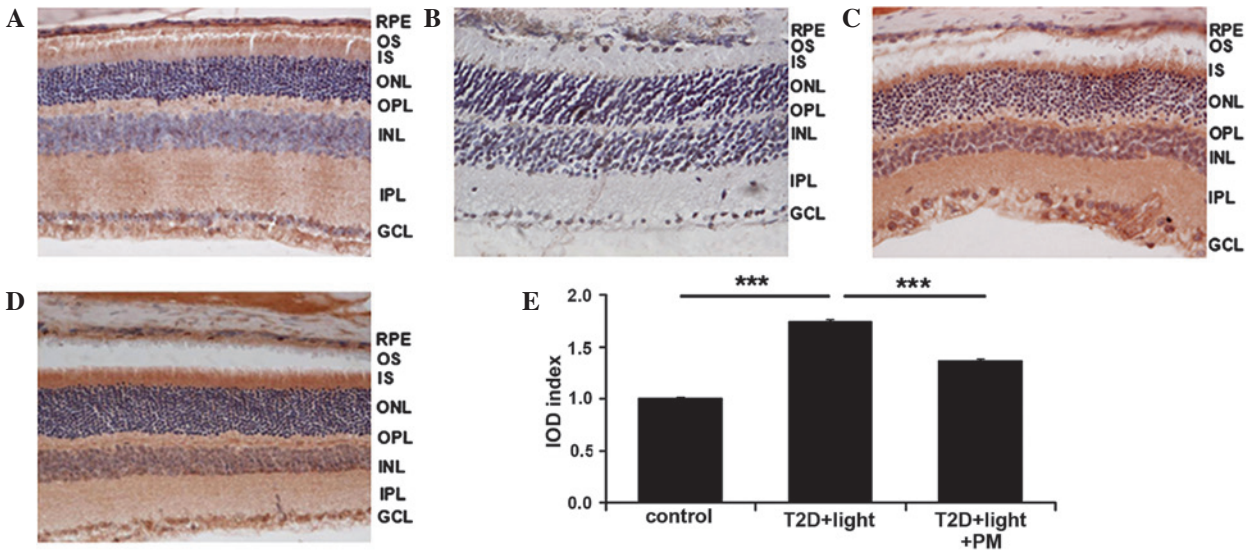

Figure 4. PM (50 mg/kg) increases the expression of antioxidant-associated proteins in the retinas of T2D mice. Immunohistochemical staining of retinal sections for apoptosis signal-regulating kinase 1 (ASK1) from (A) the control, (B) PBS treatment, (C) T2D + light treatment and (D) T2D + light + PM treatment groups. Magnification, x400; 3,3'-diaminobenzidine staining. (E) IOD was measured and demonstrated that PM treatment significantly decreases the protein expression levels of ASK1. Data are presented as the mean ± standard error of the mean, repeats = 3. ***P<0.001. RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; IOD, integrated optical density; T2D, type 2 diabetes; PM, pyridoxamine.

Results

FBG, homeostatic model assessment-insulin resistance (HOMA-IR) and establishment of the T2D mouse model. FBG levels and HOMA-IR were significantly increased 5 days after STZ injection (P<0.05). The T2D animal model was successfully constructed at day 20 after STZ injection, when FBG levels reached 11.1 mmol/l (Fig. 1).

PM protects the retinal photoreceptor cells of mice with T2D. To investigate the protective effects of PM on the retinal photoreceptor cells of T2D mice, T2D mice were treated with different concentrations of PM (25, 50 or 100 mg/kg). Following treatment, morphological observation was conducted in all groups. The mean total number of photoreceptor cells, measured at equidistant loci of the superior and inferior retina, was decreased in T2D mice compared with the control mice (P<0.001), whereas the numbers in the PM-treated groups were increased compared with the T2D + light group (P<0.001; Fig. 2A and B). To explore this change in association with gene expression levels, RT-qPCR was performed to measure Trx mRNA expression. Trx mRNA expression levels were significantly decreased in the T2D + light group compared with control mice (P<0.001), whereas, compared with T2D+ light mice, Trx expression was significantly increased following PM treatment (25 mg/kg, P<0.05; 50 or 100 mg/kg, P<0.001; Fig. 2C).

PM effects the expression of signalling pathway proteins. To explore the protective effects and mechanism of PM, western blotting and immunohistochemistry were performed to detect the expression of p-Erk1/2 and Nrf2, and ASK1, respectively. It was observed that p-Erk1/2 and Nrf2 protein expression levels were significantly increased following PM treatment compared with the levels in untreated T2D + light mice (P<0.01; Fig. 3). By contrast, ASK1 expression levels were significantly decreased following PM treatment compared with untreated T2D + light mice (P<0.001; Fig. 4). The results of the present study indicate that PM can protect retinal photoreceptor cells in T2D mice and that the mechanism of PM is associated with the upregulation of p-Erk1/2 and Nrf2 expression, and the downregulation of ASK1 expression.

Discussion

Since 1966, when Noell established the rat retinal light damage animal model, researchers have used different types of animals and light conditions to cause retinal damage (11). The light damage model is a well established animal model for the analysis of retinal degeneration diseases (11). As the development of DR is a slow process, the present study employed a light damage model to accelerate the process during experimentation.

The present study observed that a decrease in the number of retinal photoreceptor cells following STZ injection continued over time. The data demonstrated that DR is a neurodegenerative disease. Additionally, DR is understood to be a multifactorial disease that involves a variety of signalling pathways and active substances. Thus, it is important to identify novel preventive and therapeutic methods for controlling DR.

A number of studies have reported the existence of pyridoxine (P) deficiency in T1D and T2D patients, and in experimental diabetes models (12,13). P is rapidly taken up by red blood cells, and converted to PM and pyridoxal phosphate (PP). P, PP and PM form the vitamin B6 compound group and are interconvertible within the cell. The biochemical mechanism by which PM exerts its beneficial effects against cellular damage in diabetes is unclear. Thus, the present study used PM to treat T2D mice to investigate the mechanisms of action.

The mitogen-activated protein kinase (MAPK) signalling pathway is comprised of three subfamilies: c-Jun N-terminal kinase, Erk1/2 and p38MAPK. These pathways are known to be activated by oxidative stress. Deviation from the strict regulation of MAPK signalling via oxidative stress can cause the development of human diseases, including various neuro-degenerative diseases, DM and cancer (9). Furthermore, the activity of ASK1, a member of MAPK kinase kinase family, is regulated by Trx.

Erk1/2 are a major MAPK subfamily and their signalling is recognized as an important pathway in the transduction of extracellular signals to induce cellular responses. Additionally, Erk1/2 are involved in various physiological effects and pathological processes (14,15). Numerous cellular activities, including proliferation, differentiation and development, are associated with the Erk1/2 signalling pathway (16). However, inappropriate and continuous pathway activation contributes to oncogenesis, diabetic complications and angiogenesis (17). In previous studies, sulforaphane (SF), an inducer of phase II detoxification enzymes and an inhibitor of phase I enzymes, has been demonstrated to inhibit retinal degeneration (9). Furthermore, increased expression of Nrf2, a transcription factor that binds to the antioxidant responsive element, also protects retinal cells from oxidative stress by inducing phase II enzymes (5). Trx induction is mediated by Erk signalling. The Erk signalling pathway has previously been demonstrated to be involved in the SF-mediated upregulation of Trx/Trx reductase 1/Nrf2 expression *in vivo* (9). Thus, the present study investigated the expression of proteins associated with this pathway. The data of the current study demonstrated that PM significantly increases the expression of Trx, Nrf2 and p-Erk1/2 compared with untreated T2D mice. These data indicate that PM exerts effects similar to those of antioxidant enzymes by upregulating the expression of a phase II enzyme (Trx) and Nrf2.

In conclusion, the results of the present study demonstrated that PM protects retinal photoreceptor cells in T2D mice exposed to light damage and that its protective mechanism of action may be associated with the upregulation of Trx, p-Erk1/2 and Nrf2, and downregulation of ASK1.

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