

Nuclear PKR in retinal neurons in the early stage of diabetic retinopathy in streptozotocin-induced diabetic rats

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Abstract. Retinal neuron apoptosis is a key component of diabetic retinopathy (DR), one of the most common complications of diabetes. Stress due to persistent hyperglycaemia and corresponding glucotoxicity represents one of the primary pathogenic mechanisms of diabetes and its complications. Apoptosis of retinal neurons serves a critical role in the pathogenesis of DR observed in patients with diabetes and streptozotocin (STZ)-induced diabetic rats. Retinal neuron apoptosis occurs one month after STZ injection, which is considered the early stage of DR. The molecular mechanism involved in the suppression of retinal neuron apoptosis during the early stage of DR remains unclear. RNA-dependent protein kinase (PKR) is a stress-sensitive pro-apoptotic kinase. Our previous study indicated that PKR-associated protein X, a stress-sensitive activator of PKR, is upregulated in the early stage of STZ-induced diabetes. In order to assess the role of PKR in DR prior to apoptosis of retinal neurons, immunofluorescence and western blotting were performed to investigate the cellular localization and expression of PKR in the retina in the early stage of STZ-induced diabetes in rats. PKR activity was indirectly assessed by expression levels of phosphorylated eukaryotic translation initiation factor 2 α (p-eIF2- α) and the presence of apoptotic cells in the retina was investigated by TUNEL assay. The findings revealed that PKR was localized in the nucleus of

retinal ganglion and inner nuclear layer cells from normal and diabetic rats. To the best of our knowledge, the present study is the first to demonstrate nuclear localization of PKR in retinal neurons. Immunofluorescence analysis demonstrated that PKR was expressed in the nuclei of retinal neurons at 3 and 6 days and its expression was decreased at 15 days after STZ treatment. In addition, p-eIF2- α expression and cellular localization followed the trend of PKR, suggesting that this pro-apoptotic kinase was active in the nuclei of retinal neurons. These findings are consistent with the hypothesis that nuclear translocation of PKR may be a mechanism to sequester active PKR, thus preventing upregulation of cytosolic signalling pathways that induce apoptosis in retinal neurons. Apoptotic cells were not detected in the retina in the early stage of DR. A model was proposed to explain the mechanism by which apoptosis of retinal neurons by PKR is suppressed in the early stage of DR. The possible role of mitochondrial RNA (mtRNA) and *Alu* RNA in this phenomenon is also discussed since it was demonstrated that the cellular stress due to prolonged hyperglycaemia induces the release of mtRNA and transcription of *Alu* RNA. Moreover, it mtRNA activates PKR, whereas *Alu* RNA inhibits the activation of this protein kinase.

Introduction

Diabetic retinopathy (DR) is one of the most common complications of diabetes and the leading cause of vision loss globally (1,2). It is estimated that diabetes will affect ~642 million people worldwide by 2035 (3) and nearly all patients with type 1, and more than half with type 2 diabetes, will develop DR (1). DR pathophysiology is complex and involves several mechanisms, such as oxidative stress, mitochondrial dysfunction, an increase in inflammatory mediators and metabolic abnormalities initiated by hyperglycaemia (4-6). Apoptosis of retinal neurons serves a critical role in the pathogenesis of DR observed in patients with diabetes and streptozotocin (STZ)-induced diabetic rats (5,7-9). Retinal neuron apoptosis occurs one month after STZ injection, which is considered the early stage of DR (6,10). The molecular mechanisms involved in the suppression of retinal neuron apoptosis during the early stage of DR remain unclear.

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Abbreviations: PKR, RNA-dependent protein kinase; RAX, PKR-associated protein X; mtRNA, mitochondrial RNA; SINE, short interspersed nuclear elements

Key words: diabetic retinopathy, nuclear PKR, retinal neuron, apoptosis

RNA-dependent protein kinase (PKR) is a serine/threonine-protein kinase implicated in the modulation of the stress response and pro-inflammatory pathways, serving a role in the regulation of critical cell processes (11), including the apoptosis of retinal ganglion cells (RGCs) and neurons in patients with Parkinson's, Alzheimer's and Huntington's disease (12-16). PKR is ubiquitously expressed in mammalian cells and was initially identified by its activation by double-stranded RNA (dsRNA) intermediates during viral infection via a mechanism involving autophosphorylation (11,17). PKR serves an essential role in cell antiviral defence; studies on its activation have been performed primarily with viral and synthetic dsRNA (18-20). Single-stranded RNAs containing secondary structure motifs activate PKR (14,19). Mitochondrial RNA (mtRNA) is present as dsRNA in the cytosol where it interacts with PKR, resulting in PKR autophosphorylation, especially under stress (21).

Notably, the activation of PKR is stress responsive and its induction results in inhibition of cell proliferation, suppression of translation and induction of apoptosis (15,22,23). PKR serves a role in endoplasmic reticulum (ER) stress-dependent apoptosis via the eukaryotic translation initiation factor 2 α (eIF2- α)/activating transcription factor 4/CHOP signalling pathway (15) and ER stress has a role in the early stage of DR (24).

PKR-associated protein X (RAX), a direct activator of PKR, induces apoptosis by activating the PKR signalling pathway under stress (25). Our previous study (26) showed that RAX expression is indirectly modulated by microRNA (miR-29b) at the early stage of STZ-induced diabetic rats. This finding suggests that miR-29b serves a protective effect against apoptosis of retinal neurons via the PKR signalling pathway. The present study investigated the potential mechanisms underlying protection of retinal neurons against apoptosis by PKR in the early stage of DR. The present study investigated the subcellular localization, expression levels and activity of PKR in the retina of normal and STZ-induced diabetic rats.

Materials and methods

Animals and STZ treatment. A total of 168 Wistar rats (male; weight, 130-150 g; age, 5-6 weeks) were obtained from the Animal Center of School of Medicine, University of São Paulo, São Paulo Brazil. The animals were housed in suspended wire-bottom cages under environmentally controlled temperature (25 \pm 2°C) and humidity (55 \pm 5%) in a 12/12 h dark/light cycle. The animals were provided food and water *ad libitum*. Animals were maintained under these conditions for \geq 4 days prior to the experiments. The animals were randomly divided into seven groups (n=8 rats/group): Control group and six experimental groups, in which animals were killed 3, 6, 15, 22, 28 and 35 days after STZ injection. Experimental diabetes mellitus was induced by single intravenous injection of STZ (Sigma-Aldrich; Merck KGaA) dissolved in 0.01 M citrate buffer (pH, 4.5) within 5 min of preparation. The rats were fasted overnight, anesthetized with 5% isoflurane until loss of righting reflex and were immediately injected with STZ in the jugular vein at a dose of 45 mg/kg body weight. Control rats received only citrate buffer.

At 24 h after STZ administration, blood glucose levels were measured via the colorimetric oxidase glucose method

(Labtest) and animals with a blood glucose value \geq 400 mg/dl were used in subsequent experiments (27). All experiments were performed between 8:00 and 10:00 a.m. STZ-injected and control rats were sacrificed by rapid cervical dislocation. The death of animals was confirmed by observing cardiac and respiratory arrest for 3-5 min before retinas were dissected and used for analysis of PKR and p-eIF2- α expression levels or immunofluorescence. The care and treatment of the animals received prior institutional approval from the Ethical Commission on Animal Research of the School of Medicine at the University of São Paulo.

TUNEL apoptosis assay. Apoptotic cells in the retina were investigated using a DeadEnd™ Colorimetric TUNEL system (Promega Corporation) according to the manufacturer's protocol. Briefly, slides were fixed in 4% formaldehyde for 15 min at room temperature and then washed with PBS before permeabilization with protein k (10 ng/ml for 15 min). The slides were rewashed in PBS, fixed in 4% paraformaldehyde for 15 min at room temperature and immersed in PBS. Both test and negative control slides were covered with buffer solution (Promega Corporation) while the positive control reaction was performed. For the positive control, DNase I and its respective buffer (Promega Corporation) were added for 10 min at 37°C in a humid chamber. The negative control was performed using RNase-free water instead of DNase. The slides were then washed in 2X saline-sodium citrate buffer diluted in deionized water (1:10), incubated for 5 min at room temperature in 0.3% hydrogen peroxide, diluted in PBS and washed twice with PBS (5 min each). Horseradish peroxidase (HRP)-labelled streptavidin solution was prepared at a 1:500 dilution in PBS. Each slide received 100 μ l streptavidin-HRP and was incubated for 5 min at room temperature followed by washing in PBS. Finally, the slides were incubated in 1X DAB solution (DAB 10X chromogen in DAB substrate 1X Buffer) for 10 min in a humid dark chamber at room temperature. The slides were dehydrated, mounted in Permount (Thermo Fisher Scientific, Inc.) and examined under a light microscope (Leica DFC 340 FX; Leica Microsystems GmbH) at an original magnification of x100.

Immunofluorescence. Enucleated eyes were fixed using 4% buffered paraformaldehyde dissolved in 0.2 M phosphate buffer (pH 7.3) for 24 h at 4°C, then treated with 70% ethanol (2 h), 99% ethanol (1 h), absolute ethanol (30 min) and xylene (1 h) at room temperature, followed by treatment with xylene for 10 min at 37°C before being placed in a paraffin bath (1 h; 56°C). The fixed samples were subjected to vacuum pressure for 30 min and then embedded in paraffin (2 baths at 60°C, 2 h each). The paraffin blocks were cut into 5- μ m serial sections and placed on Superfrost™ (Cole-Parmer Ltd.; Thermo Fisher Scientific, Inc.) slides. In order to confirm cellular integrity, the samples were stained using Harris hematoxylin and eosin-phloxine. Sections were adhered to the slides in an oven at 80°C for 15 min, deparaffinized in xylene and rehydrated via graded ethanol washes (100, 95, 80, and 70%) for 10 min each, followed by washing in distilled water for 5 min. Sections were stained at room temperature with 5% w/v hematoxylin in water for 1 min, washed in distilled water and stained with 0.5% w/v eosin in water for 2 min. Samples were blocked at room temperature in 1% goat serum, 2% BSA and 0.05% Triton X-100 in PBS for

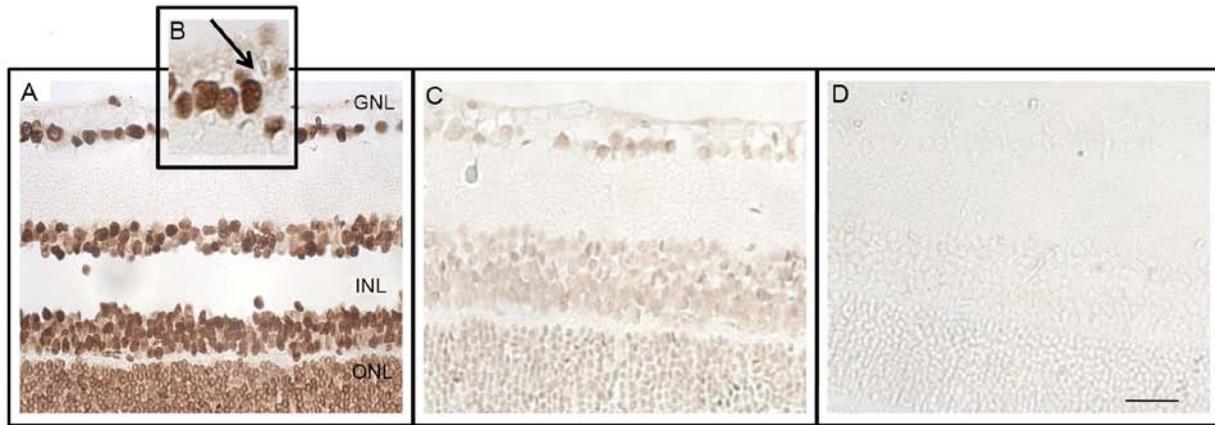


Figure 1. TUNEL staining of retinal tissues in normal and diabetic rats. Representative photomicrographs of retinal sections were evaluated for apoptosis by TUNEL assay at day 35. (A) Photomicrograph of the retina of a diabetic animal from day 35 treated with DNase I (positive control). (B) Magnification of the GCL of the positive control shows condensed and fragmented nuclei and TUNEL-positive cells. (C) Photomicrograph of the retina of a diabetic animal 35 days after treatment with streptozotocin. (D) Negative control (absence of terminal deoxynucleotidyl transferase recombinant enzyme). Magnification, 100x. Scale bar, 50 μ m. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

1 h, then incubated with anti-PKR mouse monoclonal (1:50; cat. no. sc-6282; Santa Cruz Biotechnology, Inc.) or anti-p-eIF2- α rabbit polyclonal antibodies (1:50; cat. no. 44-728G; Thermo Fisher Scientific, Inc.) for 3 h at 4°C. The samples were washed for 15 min in PBS and incubated with species-specific fluorescent Alexa Fluor 594 rabbit anti-mouse or goat anti-rabbit (H+L) (both 1:2,000; cat. nos. A-11062 and A-11037, respectively; both Thermo Fisher Scientific, Inc.) in a humidified chamber at room temperature for 1 h in the dark. Then, slides were mounted in Vectashield Mounting Medium with DAPI (Sigma-Aldrich; Merck KGaA) and examined under a microscope (magnification, x100; Leica DFC 340 FX). Images were analyzed using Adobe PhotoShop CS6 version 13.0x 64 software (Adobe Systems, Inc.). The specificity of antibody staining was confirmed by incubating adjacent sections in the absence of the primary antibody.

Western blot analysis. Total protein was extracted from rat retina using lysis buffer [20 mM Tris-HCl (pH 7.6), 400 mM NaCl, 50 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, 1 mM dithiothreitol, 1 mM EDTA, 1% Triton X-100 and 20% glycerol]. All reagents were purchased from Sigma-Aldrich (Merck KGaA). The homogenates were centrifuged at 15,500 x g at 4°C for 40 min and the supernatant was used for analysis. The protein concentrations were determined using Pierce BCA Protein Assay (Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 μ g) were separated by electrophoresis using a 10% SDS-PAGE resolving gel with 4% SDS-PAGE stacking gel and transferred onto a Hybond-C-supported nitrocellulose membrane (Amersham Biosciences; Cytiva). The membranes were subsequently blocked with 3% BSA in 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Tween-20 (TBST) for 1 h at 4°C with gentle agitation. After washing in TBST for 20 min, the membranes were incubated overnight with anti-PKR (1:300; cat. no. sc-6282; Santa Cruz Biotechnology, Inc.) and anti- β -actin mouse monoclonal antibodies at 4°C (1:1,000; cat. no. sc-517582; Santa Cruz Biotechnology, Inc.). The membranes were subsequently washed with TBST for 20 min and incubated with

HRP-conjugated anti-mouse IgG κ binding protein (1:5,000; cat. no. sc-516102; Santa Cruz Biotechnology, Inc.) antibodies diluted in TBST at room temperature for 60 min. Membranes were washed for 20 min with TBST. Antibody-labeled protein bands were visualized with enhanced chemiluminescence detection reagents (Amersham Biosciences; Cytiva) according to the manufacturer's protocol. The films were observed and the intensity of protein bands was determined using ImageJ v1.4.1 software (National Institutes of Health).

Statistical analysis. Data are presented as the mean \pm SD (n=3) and were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Statistical analysis was performed using GraphPad Prism software (version 4.0; GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

TUNEL apoptosis assays. The *in situ* presence of apoptotic cells in the sections of control and treated rat retina following STZ injection on days 6, 15, 22, and 35 was investigated by the TUNEL method. The morphological patterns obtained in the positive control, such as condensed and fragmented nuclei and the presence of apoptotic bodies, were compared with retinal cells of the diabetic and negative groups (Fig. 1A and B). At 35 days after treatment with STZ (Fig. 1C), there was no increase in the number of apoptotic cells, indicating that the present study evaluated molecular events that preceded apoptosis of neurons in the retina of diabetic rats. The negative control is shown in Fig. 1D.

Expression levels of PKR protein. Western blot analysis showed that PKR protein expression levels did not change on days 3 and 6 after STZ treatment compared with the control (Fig. 2). However, on days 15, 22, 28 and 35, a significant decrease in PKR protein expression levels was observed.

Cellular localization of PKR. Cellular localization of PKR was investigated in the retina of normal and diabetic rats on days 3, 6

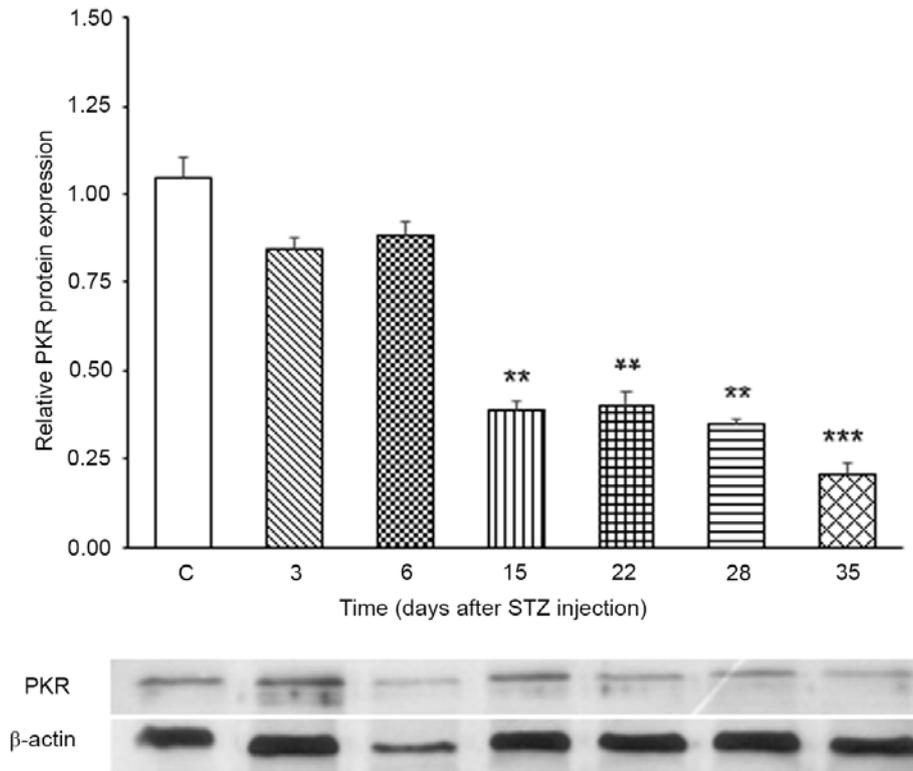


Figure 2. PKR protein expression levels in the retina of normal and diabetic rats. PKR protein expression levels were evaluated by western blot analysis of retinas of normal and diabetic rats. Data are presented as the mean \pm SEM (n=3). **P<0.01, ***P<0.001 vs. C. PKR, RNA-dependent protein kinase; C, control; STZ, streptozotocin.

and 35 after STZ injection (Fig. 3). PKR staining was observed in the nuclei of RGCs and INL cells. No change was observed in the cellular localization of PKR in the retinas of normal or diabetic rats. However, expression levels of nuclear PKR in the retinas of diabetic rats were more evident on days 3 and 6 (Fig. 3B and C) compared with control animals (Fig. 3A). Minimal expression was detected at 35 days after STZ injection (Fig. 3D). The DAPI-stained nuclei are shown in Fig. 3E-H and the negative controls in Fig. 3I-L. PKR protein nuclear staining on day 6 after STZ injection is presented in Fig. 4.

Cellular localization of p-eIF2- α . Activated eIF2- α protein (p-eIF2- α) was expressed in the same retinal neurons where PKR was detected from the retina observed for PKR (Fig. 5). In addition, the number of p-eIF2- α -positive retinal cells was the same in the normal and diabetic groups (Fig. 5A-C). Nuclear expression of p-eIF2- α in RGCs and the inner nuclear layer (INL) was observed at 3 and 6 days. Moreover, at 35 days after STZ injection, p-eIF2- α expression levels were decreased in the retina of treated rats compared with controls (Fig. 5A and D). The DAPI-stained nuclei are shown in Fig. 5E-H and the negative controls in Fig. 5I-L.

Discussion

The present study evaluated the subcellular localization, expression levels and activity of the pro-apoptotic kinase PKR in retinal neurons during the first 35 days after STZ injection, which is considered the early stage of DR in this experimental model of type 1 diabetes (28). The first step was to investigate

PKR expression levels and subcellular localization in the retina of control and STZ-treated rats. PKR protein expression levels were downregulated at 15, 22, and 28 days and PKR was localized in the nuclei of RGCs and INL cells in the rat retina. To the best of our knowledge, this is the first demonstration of the nuclear localization of PKR in retinal neurons. The activation of PKR was indirectly assessed by eIF2- α phosphorylation, as previously reported (29). A strong signal of p-eIF2- α -positive cells was observed up to 22 days after STZ treatment compared with normal retinas. In addition, analysis of cellular localization of p-eIF2- α showed that this protein was strongly expressed in the nucleus of neurons of the GCL in the retina of diabetic animals, suggesting that nuclear PKR was active in retinal neurons of diabetic rats.

PKR is stress-sensitive and considered to be one of the most important pro-apoptotic kinases (15,22,23). Thus, cellular stress due to glucotoxicity in the early stage of DR induced by STZ may activate PKR and consequently induce apoptosis of retinal neurons. However, the present study did not detect apoptosis in retinal neurons in spite of the presence of active PKR in these cells.

The accumulation of active PKR in the nucleus has been proposed as a cellular stress response (30,31). It has been linked to several types of pathology, such as leukaemia development (30), radiation resistance in lung cancer (32), sporadic Alzheimer's disease (33), Creutzfeldt-Jakob disease (34) and myelodysplastic syndrome (31). The accumulation of active PKR has also been reported in the nuclei of cells treated with tunicamycin, an ER stress inductor, and in brain neurons of patients with Parkinson's and Huntington's disease (12,35). The

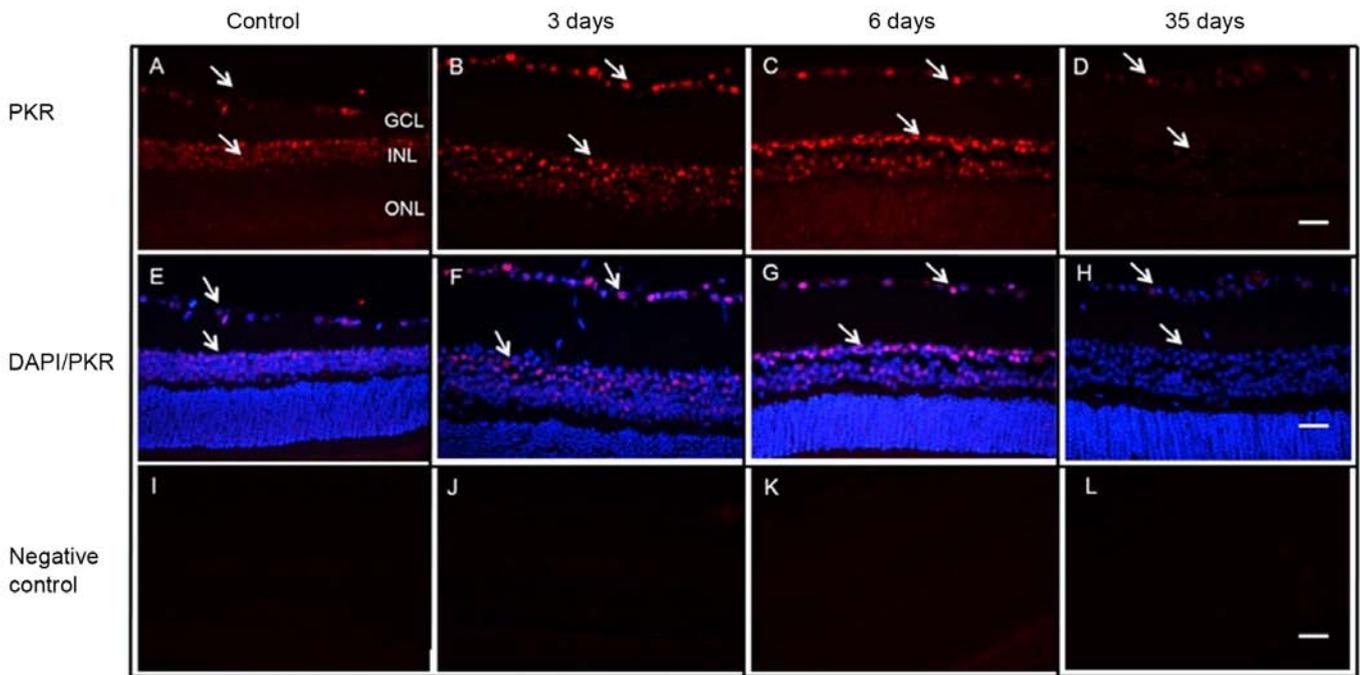


Figure 3. Immunofluorescence analysis of PKR cellular localization in the retina of normal and diabetic rats. Sections of (A) normal and diabetic rat retinas at (B) 3, (C) 6 and (D) 35 days. DAPI-stained nuclei in (E) normal and diabetic rat retinas at (F) 3, (G) 6 and (H) 35 days. Negative control of (I) normal and diabetic rat retinas at (J) 3, (K) 6 and (L) 35 days. PKR protein expression is indicated by arrows. Magnification, 100x. Scale bar, 50 μ m. PKR, RNA-dependent protein kinase; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

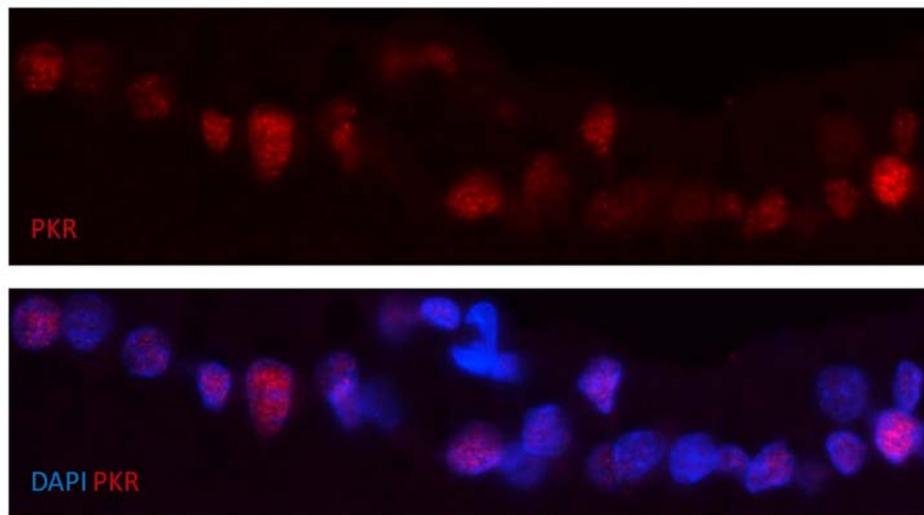


Figure 4. Immunofluorescence analysis of nuclear localization of PKR in the ganglion cell layer of the retina of diabetic rats. PKR protein nuclear staining contrasted with nuclear marker DAPI in retinal ganglion cells on day 6 after streptozotocin injection. Magnification, x400. PKR, RNA-dependent protein kinase.

biological significance of PKR translocation to the nucleus is not entirely understood. Blalock *et al* (30) reported that PKR is phosphorylated in patients with myelodysplastic syndrome and its subcellular localization depends on disease severity. Their findings suggested that nuclear translocation of PKR may be a mechanism to sequester active PKR, thus preventing this stress kinase from activating signalling pathways in the cytosol.

Previous work has revealed that cellular stress due to hyperglycaemia is responsible for mitochondrial dysfunction (36). It

was also demonstrated that mtRNA is released into the cytosol and forms intermolecular dsRNAs that activate cytoplasmic PKR (21,37). Moreover, it has been shown that different types of cellular stress, including that induced by hyperglycaemia, can induce transcription of *Alu* elements that belong to the short interspersed nuclear elements (SINE) family and are present at more than one million copies in the human genome (38,39). *Alu* RNA inhibits cytoplasmic PKR, suggesting a functional role for mammalian SINEs, which have previously been considered junk DNA (40). Accumulating evidence supports the

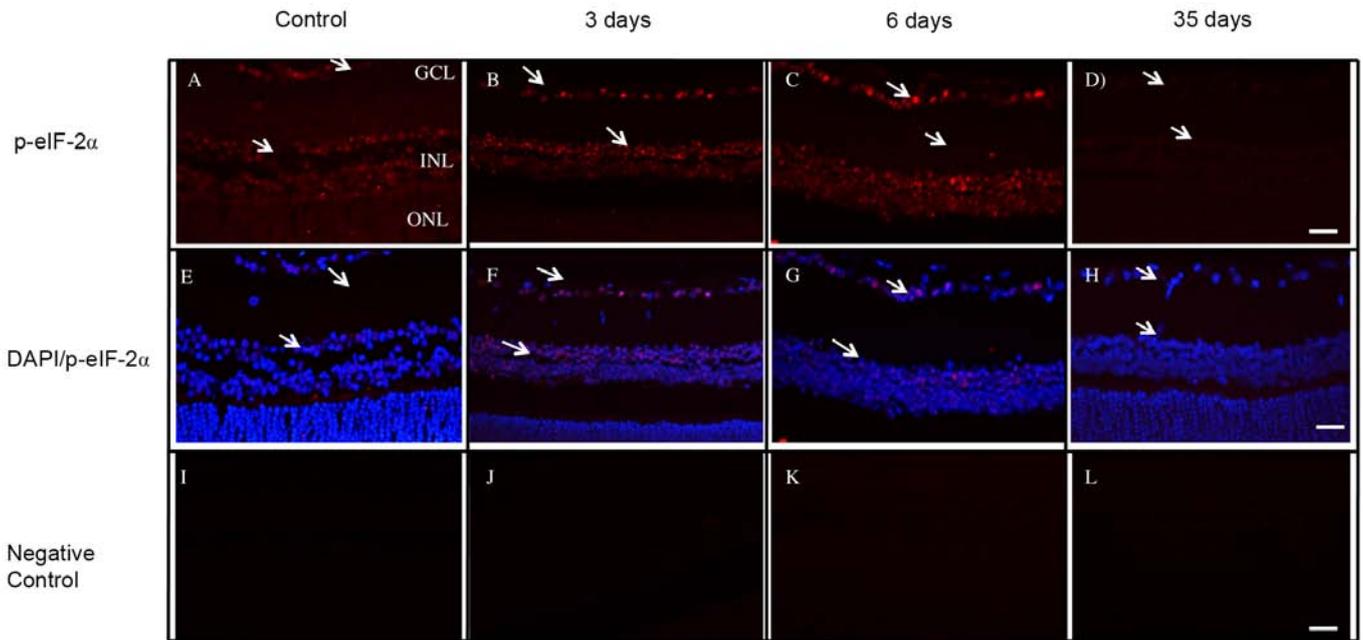


Figure 5. Immunofluorescence analysis of p-eIF2- α cellular localization in the retina of normal and diabetic rats. Sections of (A) normal and diabetic rat retinas at (B) 3, (C) 6 and (D) 35 days. DAPI-stained nuclei in the retina of (E) normal and diabetic rats at (F) 3, (G) 6 and (H) 35 days. Negative controls of (I) normal and diabetic rat retinas at (J) 3, (K) 6 and (L) 35 days. Arrows indicate p-eIF2- α protein. Magnification, x100. Scale bar, 50 μ m. p-eIF2- α , phosphorylated eukaryotic translation initiation factor 2 α ; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

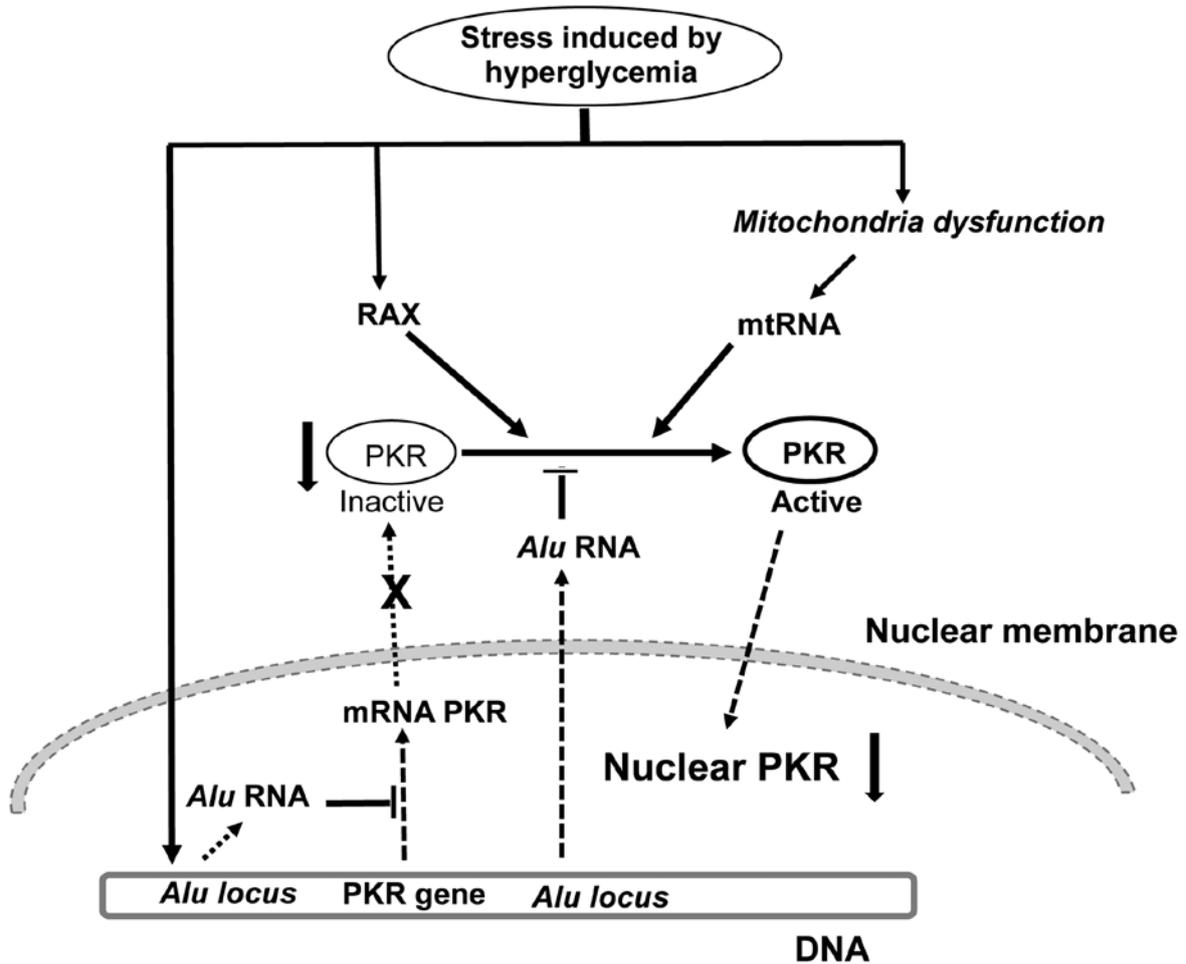


Figure 6. Overview of the proposed mechanism underlying downregulation of nuclear PKR expression in retinal neurons in the early stage of diabetic retinopathy in streptozotocin-treated animals. PKR, RNA-dependent protein kinase; RAX, PKR associated protein X; mtRNA, mitochondrial RNA; *Alu*, a class of retroelements termed short interspersed nuclear elements.

concept that *Alu* RNAs serve a functional role during the stress response. *Alu* RNA inhibits the transcription of genes involved in the response to heat shock (39) and can inhibit activation of the cytoplasmic PKR (40). The induction of *Alu* element transcription in hyperglycaemic endothelial cells has been observed, which is responsible for inhibition of the synthesis of nitric oxide synthase and superoxide dismutase 2 (41).

Based on the present results and the literature, we proposed a mechanism to explain the decrease in nuclear PKR expression levels in retinal neurons at 35 days after STZ treatment (Fig. 6). We hypothesize that stress caused by hyperglycaemia induces RAX expression (26) and mitochondrial dysfunction, with subsequent release of mtRNA into the cytosol of neurons (21,37). RAX and mtRNA activate PKR in the cytosol of retinal neurons, which is followed by its nuclear translocation. Nuclear translocation of active PKR has previously been suggested as a cell protection mechanism against the pro-apoptotic activity of PKR (31). Stress caused by hyperglycaemia induces the expression of *Alu* RNAs (41), which inhibit transcription of the PKR gene and activation of PKR (39), leading to a decrease in nuclear PKR protein expression levels in retinal neurons of STZ-induced diabetic animals. Thus, the present results suggest a novel mechanism that may underly the protection of retinal neurons from apoptosis by PKR at the early stage of DR, in addition to the inhibition of RAX expression by miR-29b, as previously described (26). Bone marrow mononuclear cells of high-risk patients with myelodysplastic syndrome display nuclear localization of active PKR and a lower number of apoptotic cells compared with low-risk patients (31). In conclusion, the present study may contribute to understanding of the biological significance of PKR nuclear translocation observed in pathologies, such as myelodysplastic syndrome (31) and Alzheimer's (33) and Creutzfeldt-Jakob disease (34).

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

VAOS and FLDL designed the experiments and wrote the manuscript. VAOS, NDA, TAES, IDCK and VMA performed the experiments. VAOS and NDA analyzed the data. All authors discussed the data and commented on the manuscript.

VAOS, NDA and FLDL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Commission on Animal Research of the School of Medicine at the University of São Paulo (approval no. 012/2008).

Patient consent for publication

Not applicable.

Competing interests

The authors confirm that they have no competing interests.

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