Original Article

ASK1 induces retinal microvascular endothelial cell apoptosis through ER stress-associated pathway

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Abstract: Diabetic retinopathy (DR) is a major microvascular complication in patients with diabetes mellitus; it can cause a variety of eye problems in a high percentage of diabetic patients. The purpose of this study was to determine the role of apoptosis signal-regulating kinase 1 (ASK1) in the regulation of the endoplasmic reticulum (ER) stress-associated apoptosis pathway in microvascular endothelial cells. For in vivo studies, a streptozotocin (STZ)-induced diabetes model was used to assess apoptosis in retinal tissues. Apoptotic cell death was determined by TUNEL assay. For in vitro studies, a high glucose (HG)-induced retinal microvascular endothelial cell injury model was generated to evaluate apoptosis. Apoptotic rates were measured by flow cytometry and apoptosis-related proteins were detected by western blotting. We found that retinal microvascular endothelial cell apoptosis was increased in both animal and cell models. HG-induced apoptosis primarily occurred in an ER stress-dependent manner. HG-induced apoptosis was alleviated by inhibiting ASK1 with shRNA or a specific inhibitor, NQDI-1. TUNEL and western blot assays showed that ASK1 promoted the expression of ER stress-related proteins that are the master regulators of DR. Our study suggests that ASK1 functions as a promoter of DR through the ER stress-induced apoptosis pathway, and it may be a therapeutic target for DR.

Keywords: ASK1, diabetic retinopathy, ER stress, invasion, apoptosis

Introduction

Diabetic retinopathy (DR) is a chronically progressive, potentially vision-threatening disease of the retinal microvasculature; it is a major cause of blindness among working-age individuals [1]. Chronic hyperglycemia-associated low-grade aseptic inflammation is thought to play a key role in the initiation and progression of DR [2-4]. Despite a vast amount of investigative effort, the pathogenesis of DR remains unclear. It has been reported that the endoplasmic reticulum (ER) stress-associated apoptosis pathway is involved in DR progression [5-7]. However, the regulatory role of ASK1 on DR occurrence has not been reported.

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase (MAPK) kinase family that activates the p38 MAPK and c-Jun NH2-terminal kinase signaling pathways in response to various stimuli, including oxidative stress, endoplasmic reticulum stress, infection and calcium influx [8, 9]. Under these stress conditions, ASK1 plays pivotal roles in cellular signaling pathways and biological functions. Hyperglycemia increases oxidative stress in various tissues, and it has been reported that high glucose-induced activation of ASK1 contributes to endothelial cell senescence, leading to diabetes-related vascular aging mediated by oxidative stress [10]. Previous studies have shown that ASK1 plays a regulatory role in diabetes complications, including diabetic cardiomyopathy, diabetic kidney disease, and diabetic embryopathy [11-13]. Nevertheless, the effect of ASK1 on DR has not yet been elucidated. Therefore, we investigated whether ASK1 contributed to high glucose-induced apoptosis of retinal microvascular endothelial cells.

The ER is a critical intracellular organelle, with several vital functions including protein trans-
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port and protein synthesis, and it acts as a reservoir for \( \text{Ca}^{2+} \) [14]. There is evidence suggesting that ER stress can lead to pancreatic \( \beta \)-cell apoptosis, inhibition of insulin receptor expression, and insulin resistance, and ultimately the development of type 2 diabetes [2, 15]. A study reported that ER stress in cultured human retinal pericytes was increased after hypoglycemia and when glucose concentrations were reduced from high to low levels. Apoptotic pathways are ultimately induced if ER stress cannot be relieved by protein folding and degradation [16]. While it has been suggested that early pathological changes associated with DR may involve apoptosis, it is not clear whether ASK1 is involved in ER stress-induced apoptosis.

In the present study, we first found that ASK1 promoted ER stress-induced apoptosis in DR. We therefore sought to clarify the contribution of the ER stress-mediated apoptosis signaling pathways to the high glucose-induced inflammatory response and apoptosis using human retinal microvascular endothelial cells (HRMECs) and to assess the protective effect of NQDI-1 on hyperglycemia-induced injury.

Materials and methods

Animal model

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 Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. Six-week-old male inbred Sprague Dawley (SD) rats weighing 100-120 g (Shanghai Laboratory Animal Center of the Chinese Academy of Sciences) were housed 5 per cage in an animal colony facility for 2 weeks. The animals were maintained in a room with a constant temperature (22 ± 2°C). All animals were born and raised in a 12-h-light/12-h-dark environment with an average illumination of 80 lx. Tap water and food pellets were provided. SD rats were randomly divided into the DM group and the non-diabetes group. The DM group was intraperitoneally injected with 60 mg/kg STZ. Tail vein blood glucose levels were measured at 48 h after the injection, and those with blood glucose ≥ 16.7 mmol/L were considered DM rats. Diabetes was induced in rats by STZ treatment for 8 weeks. The experiments were conducted between 10:00 and 14:00.

STZ (Sigma) was dissolved in cold 50 mM citric acid buffer (pH 4.5).

Cell lines and cell culture

HRMECs were purchased from Cell Systems (Kirkland, WA, USA), and cells from passages 3-7 were used in the experiments. Cells were grown in M199 medium with 45 ng/ml bFGF, heparin, 20% fetal bovine serum, and 1% penicillin-streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl) in a humidified atmosphere of 5% \( \text{CO}_2 \) and 95% air at 35°C. The passage number range for this cell line was maintained between 10 and 15. The cells were cultured in a 10 cm 2 cell culture dish; 90% confluent cells were switched to serum-free medium for 24 h before the experiment was conducted. The cells were incubated with normal glucose (5 mmol/L) plus 100 nM ASK1 inhibitor, NQDI-1 (100 nmol/L, Selleck, China) or with high glucose (30 mmol/L) plus 100 nM NQDI-1 for 48 h. NLRP3 was inhibited with 50 nmol/L MCC950 (Selleck, China).

TUNEL assay

The TUNEL method was employed to detect apoptotic cells according to manufacturer’s instructions. Briefly, cultured HRMECs were infected with lentivirus vectors expressing shScramble or shASK1 (or pretreated with 100 nM ASK1 inhibitor NQDI-1) and then incubated with low glucose (5 mM) or high glucose (30 mM) medium for 48 h. The percentage of apoptotic cells was assessed by terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method using an apoptosis in situ detection kit (DeadEnd™ Colorimetric TUNEL System, Promega, USA). After TUNEL labeling, the nuclei were labeled with 4’,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Invitrogen, Carlsbad, CA, USA). The FITC-labeled, TUNEL-positive cells were imaged under a fluorescence microscope with 488 nm excitation and 530 nm emission. After four cortical fields were randomly selected from each section, 100 cells were successively counted for each field by a blinded observer. The ratio of TUNEL-positive cells to the total cell number is shown.

MTT assay

The MTT assay is based on the protocol that was first described by Mossmann (1983). The
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assay was optimized for the cell lines used in these experiments. Briefly, for the purpose of these experiments, at the end of the incubation time, the cells were incubated for 4 h with 0.8 mg/ml of MTT dissolved in serum free medium. After washing with PBS (1 ml), DMSO (1 ml) was added, followed by gentle shaking for 10 min so that complete dissolution was achieved. Aliquots (200 µl) of the resulting solutions were transferred into 96-well plates, and the absorbance was recorded at 560 nm using a microplate spectrophotometer system (Spectra max190-Molecular Devices). The results were analyzed and presented as a percentage of the control values.

**Western blot analysis and antibodies**

Total cell extracts were obtained by lysing the cells in RIPA buffer and boiling for 5 min. Protein concentrations were measured by the Bradford assay (Bio-Rad, Hercules, CA, USA). Cellular proteins were extracted and separated on 4-10% Tris glycine/SDS-polyacrylamide gels and electrotransferred to ECL nitrocellulose membranes (#IPFL00010, Millipore). The membranes were blocked with 5% nonfat milk and incubated with specific antibodies. β-actin protein was used as the endogenous control. Antibodies against the following proteins were purchased from Cell Signaling Technology: Caspase 4 and cytochrome c. The following antibodies were purchased from Santa Cruz Biotechnology: IRE1, CHOP, ASK1 and β-actin. Immunocomplexes were visualized by ECL (Pharmacia-Amersham, Freiburg, Germany).

**Lentivirus-mediated gene silencing**

The following short hairpin RNA (shRNA) was cloned into a pGIPZ vector and used for targeting human ASK1 (Asiavector Biology, China): TGCTGTGACATGAGCGATTGGTCGATTCTACAAAGATATAGTGAAGCCACAGATTATATTCTTGTAGATTCGACCAACTGCCTACTGCCTCGGA. Lentivirus was generated by transfection of HEK293T cells with pLKO.1 or ASK1 shRNA plasmids and packaging plasmids (PSPA and PMD2G, obtained from Asiavector Biology, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, the virus-containing cell culture supernatants were collected, filtered and concentrated. The infection of HRMECs with the ASK1-lentivirus or scramble-lentivirus (MOI = 10) was performed in the presence of 5 µg/ml polybrene (Sigma-Aldrich, USA). After 48 h, the efficiency of gene silencing was determined by semi-quantitative RT-PCR and western blot.

**Flow cytometry analysis**

Cells were washed twice with PBS before the experiments. The cells were collected via centrifugation at 1000 rpm for 5 min and then stained with Annexin V/propidium iodide (PI), Annexin V-APC/7-AAD and binding buffer (KeyGEN Biotech) at room temperature for 15 min. After mixing, the samples were analyzed using a flow cytometer. The data were analyzed on BD Accuri C6.

**Statistical analysis**

Each experiment was performed at least 3 times, on independent passages, usually in triplicate. Data were analyzed by the Newman-Keuls test using Statistica software as indicated and were presented as the mean ± SEM. P < 0.05 was considered statistically significant. The results of time-lapse microscopy experiments were analyzed with the Wilcoxon test in R software.

**Results**

**Retinal microvascular endothelial cell apoptosis is frequently increased in DR tissues and cells**

We first investigated apoptosis of retinal tissues in DR rat models. The TUNEL assay was used to determine apoptosis in control and DR samples. The apoptotic rates of DR tissues were higher than those of the control group (Figure 1A). We then examined whether HG promoted apoptosis of HRMECs. As expected, HG increased the expression level of cleaved caspase-3 (CLCasp-3) after HG incubation for 48 h (P < 0.05) in HRMECs, as determined by flow cytometry and western blotting (Figure 1B, 1C). We observed that HG induced a time-dependent decrease in cell viability (P < 0.01) (Figure 1D). Taken together, these data suggest that caspase-3 is upregulated in DR, possibly contributing to DR pathogenesis.

**High glucose-induced apoptosis primarily occurred in an ER stress-dependent manner**

To further explore the role of apoptosis in the progression of DR, HRMECs were incubated with normal glucose (NG) or high glucose (HG).
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Figure 1. Retinal microvascular endothelial cell apoptosis in DR tissues and cells. A. Light microphotographs focused on the ganglion cell layer of 8-week diabetic whole mounted retina processed for TUNEL staining. Scale bar represents 100 µm. B. Representative experiment from flow cytometric analysis of caspase-3 stained HRMECs. Data are expressed as the mean ± SEM of results in three separate experiments. C. HRMECs were treated with 30 mM glucose at various times (0 h, 6 h, 24 h, 48 h, 72 h and 96 h). Cell viability was detected by MTT assay (n = 3). D. Western blotting analysis of CLCasp-3 expression in HRMECs treated with 30 mM glucose at various times (0 h, 6 h, 24 h, 48 h, 72 h and 96 h). Tubulin served as the loading control.
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Figure 2. High-glucose-induced apoptosis primarily occurred in an ER stress-dependent manner. Expression levels of caspase-4, cyto C, IRE1α and CHOP in HRMECs were examined by western blotting. Densitometric analyses of caspase-4 (A), cyto C (B), IRE1α, and CHOP levels (C), standardized against β-actin levels in the same lane. (D) Representative experiment from flow cytometric analysis of annexin-V FITC/propidium iodide stained HRMECs. (E) Column bar graph showing apoptosis in the different groups studied.
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The expression levels of caspase-4, cyto C, IRE1α and CHOP in HRMECs were examined by western blotting (Figure 2A-C). Notably, levels of caspase-4, IRE1α and CHOP, hallmarks of endoplasmic stress, were dramatically elevated, whereas levels of cyto C, a mitochondrial apoptosis marker, were not significantly altered in HG groups. We then used flow cytometry to measure apoptosis rates among ctrl, HG-induced and HG+4-PBA groups (Figure 2D). We found that ER stress-related apoptosis was the primary pathway in the high-glucose-induced HRMECs model.

**ASK1 induced apoptosis via the ER stress pathway**

To further evaluate how ASK1 regulates the apoptosis pathway in DR, we first examined ASK1 expression levels in NG and HG-induced HRMECs cells by WB. The results from the WB indicated that HG significantly increased ASK1 expression levels (Figure 3A). Then, HRMECs were incubated with LG plus NQDI-1 or HG plus NQD-1 for 48 h. We found that NQDI-1 treatment attenuated HG-mediated apoptosis and increased viability (Figure 3B). These results suggest that ASK1 alleviates, at least in part, the level of apoptosis induced by HG in vitro. Furthermore, WB was carried out to investigate whether ASK1 was involved in apoptotic ER stress in various experimental groups. As expected, levels of CHOPα and IRE1, key mediators of apoptotic ER stress, were significantly higher after treatment with NQDI-1. HG rescued ASK1-induced apoptosis through the ER stress pathway. These results confirmed that ASK1 positively regulated IRE1α and CHOP expression (Figure 3C).

**ER stress promoted DR by upregulating ASK1**

To investigate the functional role of ASK1 in the DR model, we first assessed the level of apoptosis of retinal tissues in the control, DR, and DR+4-PBA groups. The apoptotic rates of DR tissues were higher than those of the control group, and 4-PBA rescued HG-induced apoptosis (Figure 4A). The tissues were then subjected to WB assays to identify whether 4-PBA rescued the HG-induced increases of mitochondrial apoptosis. We found that treatment with 4-PBA inhibited HG-promoted apoptosis (Figure 4B, 4C).

**Discussion**

It is well-known that ER stress plays important roles in the microenvironment of diabetes mellitus (DM), including DR [17]. Here, we found that the apoptosis rate was increased in DR in vivo and in vitro, with respect to rates in the control group. Moreover, we demonstrated that restoration of ASK1 suppressed HG-promoted apoptosis, suggesting a fundamental role of ASK1 as an ER stress-specific marker in DR.

Numerous studies have shown that ASK1 was preferentially activated in response to various types of stress, including ROS, TNF-α, lipopolysaccharides and ER stress, and has potential
ASK1 functions as a promoter of diabetic retinopathy roles in a wide variety of cellular responses, including apoptosis, inflammation and differentiation [18-20]. Nevertheless, the expression and distribution of ASK1 in HRMECs has not yet been elucidated. We found that HG contributed to ASK1 activation in HRMECs. Then, we compared the injury responses and biochemical reactions in normal HRMECs and ASK1 inhibitor-treated HRMECs cultured in HG medium. We demonstrated that activation of ASK1 by HG upregulated caspase-3 and induced endoplasmic stress protein activation, promoting apoptosis in HRMECs, whereas the inhibition of ASK1 by shRNA decreased HRMEC sensitivity to HG stress.

Several studies suggested that ER stress-mediated cell death is associated with the death of pancreatic β-cells in patients with diabetes [21]. Furthermore, a recent study demonstrated that ER stress-induced apoptosis was related to changes in glucose concentration and resulted in the death of pericytes [22]. The ER is involved in the maintenance of cellular homeostasis by inducing the UPR [23]. When the number of pancreatic β-cells is decreased, ER stress is increased in the remaining pancreatic β-cells to compensate for the reduced insulin secretion leading to pancreatic β-cell dysfunction [22]. However, the molecular mechanisms underlying ASK1-mediated biological behaviors remain unclear. To comprehensively understand the effect of ASK1 on DR cells, we investigated the apoptotic propensity of DR tissues and HG-induced cells in both in vitro and in vivo models. We found that ASK1 knockdown

![Figure 4. ER stress promoted diabetic retinopathy by upregulating ASK1. (A) Light microphotographs focused on the ganglion cell layer of 8-week diabetic whole mounted retina with different treatments processed for TUNEL staining. Scale bar represents 100 µm. The expression levels of CHOP, IRE1α (B) and caspase-4 (C) in DR or DR+4-PBA treatment groups were examined by western blotting. β-actin was used as loading control.](image)
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in HG-induced cells alleviated ER stress-induced apoptosis.

Given that ASK1 is a key element in ER stress-induced cell apoptosis, levels of ER stress markers were measured in HG cells treated with an inhibitor of endoplasmic stress. 4-PBA treatment significantly decreased expression levels of IRE1α and CHOP. These results suggest that 4-PBA treatment may reverse the DR process to inhibit cell apoptosis. To address the molecular mechanisms involved in ASK1-mediated promotion of apoptosis, caspase-4, a molecule predicted to be downstream of ER stress, was selected for further study. To investigate the therapeutic potential of inhibiting ASK1 to protect HRMECs exposed to HG, we incubated HRMECs with the recently-identified ASK1 inhibitor NQDI-1 in HG, inhibiting cellular apoptosis in HRMECs. NQDI-1 significantly inhibited activation of the endoplasmic stress protein, and attenuated HG-induced apoptosis. Taken together with the previous findings, these data suggest that ASK1 serves as a pivotal mediator in regulation of DR progression.

On the basis of these studies, our data suggested that restoring ASK1 expression attenuated protein levels of IRE1α and CHOP by regulating ER stress-related apoptosis. Targeting the ASK1/CHOP interaction or rescuing ASK1 expression may be a new therapeutic application to treat DR patients in the future.

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Disclosure of conflict of interest

None.

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