Calcium mediates high glucose-induced HIF-1α and VEGF expression in cultured rat retinal Müller cells through CaMKII-CREB pathway

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Aim: To investigate the effects of high glucose (HG) medium on expression of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) in cultured rat retinal Müller cells and to determine the signaling pathways mediating the effects.

Methods: Primary cultures of retinal Müller cells were prepared from Sprague-Dawley rats, and incubated in a medium containing HG (30 mmol/L) in the presence of the membrane-permeable Ca2+ chelator BAPTA-AM (10 µmol/L) or the CaMKII inhibitor KN93 (10 µmol/L). The levels of CaMKII, p-CaMKII, CREB, p-CREB, HIF-1α, and VEGF proteins were measured with Western blotting, while HIF-1α and VEGF mRNA levels were determined using real-time RT-PCR.

Results: The stimulation of retinal Müller cell with HG for 24 h remarkably increased the expression levels of HIF-1α and VEGF. These responses were significantly inhibited in the presence of BAPTA-AM or KN93. Both BAPTA-AM and KN93 also significantly inhibited HG-induced phosphorylation of CaMKII and CREB in the cultured retinal Müller cells. Transfection of the cultured retinal Müller cells with antisense CREB oligonucleotide (300 nmol/L) was similarly effective in blocking the HG-induced increase of HIF-1α and VEGF.

Conclusion: HG-induced HIF-1α and VEGF expression in cultured rat retinal Müller cells depends on intracellular free Ca2+ and activation of CaMKII-CREB pathway. The activation of CaMKII-CREB pathway by HG may be a possible mechanism underlying the pathogenesis of diabetic retinopathy.

Keywords: diabetic retinopathy; hyperglycemia; retinal Müller cells; intracellular Ca2+; CaMKII; CREB
cells\textsuperscript{[17]} and the corresponding sense oligonucleotide sequence was 5’-CACCGGTGACTAGATGACCA-3’. After transfection with the antisense or sense oligonucleotide for 24 h, the medium was removed from the cells, serum-free DMEM with NG was added, and the cells were allowed to recover for 30 min. The transfected Müller cells were washed once with serum-free DMEM and then growth-arrested for 24 h in the same medium supplemented with either NG (5.5 mmol/L) or HG (30 mmol/L).

**Materials and methods**

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85–23, revised 1996) and was carried out with the approval of the local ethics committee/ institutional board. All chemicals were of reagent grade and purchased from Sigma Chemicals (St Louis, MO, USA), unless otherwise stated.

**Cell culture**

Primary cultures of retinal Müller cells were prepared from 5 to 7-d-old Sprague-Dawley rats (purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences) following a previously described protocol\textsuperscript{[19]} , with some modifications. Briefly, enucleated eyes were washed under sterile conditions, and the anterior portions were discarded. The retinas were isolated, chopped into 1×1 mm fragments, treated with 0.1% trypsin at 37 °C for 20 min, and then passed through mesh to remove any large retinal pieces. The strained isolates were centrifuged at 800 r/min for 5 min, and the supernatant fluid was removed. The precipitated cells were resuspended and seeded in the plastic culture flask containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mmol/L NaCl, 5 mmol/L EDTA, and 10% fetal calf serum (FBS). The cultures were maintained in 5% CO\textsubscript{2} at 37 °C. The medium was routinely replaced every 3–4 d. After 8–10 d, pure Müller cells became confluent and were used for experiments.

**Immunocytochemistry**

Cultured rat Müller cells were fixed with 4% paraformaldehyde and blocked with 2% BSA in PBS containing 0.3% Triton X-100. Slides were incubated overnight in a humidified chamber at 4°C with anti-glutamine synthetase (GS) and anti-vimentin primary antibodies (1:1000 dilution). After primary antibody incubation, cells were washed three times with PBS for 10 min each and incubated in the appropriate fluorescent conjugated secondary antibody (goat anti-mouse IgG, 1:200) for 1 h. The cells were counterstained with DAPI. Images were captured with a fluorescence microscope (Olympus, Tokyo, Japan).

**Transfection of Müller cells with antisense CREB oligonucleotides**

The CREB antisense oligonucleotide synthesis and transfection were conducted as previously described\textsuperscript{[19]} . The CREB antisense oligonucleotide sequence was 5’-TGGTCATCTAGT-3’, and the corresponding sense oligonucleotide sequence was 5’ -CACCGGTGACTAGATGACCA-3’. After transfection with the antisense or sense oligonucleotide for 24 h, the medium was removed from the cells, serum-free DMEM with NG was added, and the cells were allowed to recover for 30 min. The transfected Müller cells were washed once with serum-free DMEM and then growth-arrested for 24 h in the same medium supplemented with either NG (5.5 mmol/L) or HG (30 mmol/L).

**Qualitative real-time RT-PCR analysis**

To measure HIF-1α and VEGF mRNA expression by qualitative real-time RT-PCR, total cellular RNA was extracted from retinal Müller cells with Trizol Reagent (Invitrogen Life Technologies, Shanghai, China) and stored at -80°C. A quantitative polymerase chain reaction (qPCR) kit (DYNAamo Flash SYBR Green; Finnzymes Oy, Espoo, Finland) was used according to the manufacturer’s instructions. The primer sequences (sense/ antisense) used were as follows: HIF-1α, 5’-GACAATAGCT- TGCAGAATGTC/3’-TGCAAATCTGGTGCTGGTG-3’; and VEGF, 5’-AATGATGAAGCCCTGGAGTG-3’/5’-AAT- GCTTCTCCCGCTCTGAA-3’. The specificity of the amplification product was determined by melting curve analysis. Standard curves were generated for each gene by preparing serial dilutions of the respective cDNA templates. The relative quantities of each gene were obtained by normalizing the signals to β-actin (5’-CGAAACGGTCGGCATGT-3’/5’- CGGGCCACACGGCACCTATT-3’), and each experiment was repeated independently at least three times.

**Western blot analysis**

Approximately 3×10\textsuperscript{6} retinal Müller cells were harvested and lysed in lysis buffer containing 1% NP-40, 10 mmol/L Tris, 200 mmol/L NaCl, 5 mmol/L EDTA, and 10% glycerol plus protease inhibitors (pH 7.0). Lysates from treated cells were centrifuged at 12000 r/min for 20 min at 4°C, and the cleared supernatants were collected. The protein concentration in the supernatant was measured using the Bio-Rad (Hercules, CA, USA) DC protein assay. A 30-µg aliquot of protein from each sample was subjected to electrophoresis on 10% SDS-PAGE using a Bio-Rad mini electrotransfer slab gel apparatus. Separated proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk solution and incubated overnight with partially purified rabbit anti-CaMKII and anti-phosphoThr-286-CaMKII polyclonal antibody (Abcam; 1:500), rabbit anti-CREB and mouse anti-phospho-CREB polyclonal antibody at Ser-133 (Abcam; 1:500), rabbit anti-HIF-1α polyclonal antibody (Santa Cruz Biotechnology; 1:500), and rabbit anti-VEGF polyclonal antibody (Abcam; 1:500). The expression of β-actin (monoclonal anti-β-actin; Santa Cruz Biotechnology; 1:1000) was used as an internal control to confirm equivalent protein loading per gel lane. The immunopositive bands were visualized by the ECL system (Amersham Biosciences, Buckinghamshire, England). Each experiment was performed at least in triplicate.
Statistical analysis
Experimental data are expressed as the mean±SD. Group means were compared by one-way ANOVA followed by the Dunnett post hoc test using GraphPad Prism 4.0 (San Diego, CA) and SPSS (Statistical Package for the Social Sciences) 13.0 for Windows (SPSS, Chicago, IL). A value of P<0.05 was considered significant in all cases.

Results
Immunocytochemical characterization of cultured retinal Müller cells
The identities of the cultured rat retinal Müller cells were confirmed by immunocytochemistry using antibodies against the Müller cell markers GS and vimentin. Nuclei were stained with DAPI. Almost all cells were positive for GS and vimentin (Figures 1A–1D), indicating that the primary cultures were Müller cells.

Calcium mediates HG-induced HIF-1α and VEGF expression
Intracellular Ca²⁺ signals alter gene expression patterns by activating several nuclear transcription factors [20]. To explore the effects of [Ca²⁺], on HG-induced gene expression in Müller cells, we analyzed the expression of HIF-1α and VEGF. In vitro hyperglycemia (30 mmol/L, HG) for 24 h upregulated the expression of HIF-1α and VEGF at both the mRNA and protein levels. Furthermore, the upregulation of HIF-1α and VEGF was most likely caused by increased [Ca²⁺], at least in part, because the Ca²⁺ chelator BAPTA-AM blocked this enhanced expression, while the general Ca²⁺ ionophore A23187 upregulated HIF-1α and VEGF expression in Müller cells in NG (Figures 2A–2D). Summary data for these experi-

![Figure 1. Immunocytochemical analysis of primary Müller cell cultures. Blue: nuclear staining with DAPI (A). Red: Müller cell marker GS (B). Green: Müller cell marker vimentin (C). Merged labeling of GS, vimentin and DAPI (D).](image1)

![Figure 2. Hyperglycemia induced [Ca²⁺], dependent HIF-1α and VEGF Expression. (A, B) HIF-1α and VEGF mRNA in retinal Müller cells cultured in serum-free DMEM containing normal glucose (NG, 5.5 mmol/L), NG+D-mannitol (24.5 mmol/L), high glucose (HG, 30 mmol/L), or HG in the presence of BAPTA-AM (10 μmol/L) (HG+BAPTA-AM) or NG in the presence of A23187 (1 μmol/L) (NG+A23187) for 24 h was quantified by using real time RT-PCR. Results are expressed (relative to the NG values). (C, D) Western blot analysis of HIF-1α and VEGF protein expression in retinal Müller cells incubated in the five groups for 24 h. Equal protein loading was confirmed with the β-actin antibody. Mean±SD from nine cells per group. bP<0.05, cP<0.01 vs NG or NG+D-mannitol. fP<0.01 vs HG.](image2)
ments show that HG can enhance the expression of HIF-1α and VEGF and that this effect may be mediated by increased [Ca^{2+}] in vitro.

**HG-induced [Ca^{2+}]-dependent Serine/Threonine phosphorylation of CaMKII and CREB**

Activation of the transcription factor CREB is known to orchestrate a number of signaling and gene expression pathways involved in angiogenesis[21, 22]. The following experiments were performed to examine the downstream signaling pathway(s) through which [Ca^{2+}], signals mediated cellular responses, particularly the upregulation of the angiogenic factors HIF-1α and VEGF.

To determine whether HG can induce the threonine phosphorylation of CaMKII (p-CaMKII) and the serine phosphorylation of CREB (p-CREB), retinal Müller cells were incubated for 24 h in serum-free DMEM containing NG, NG+D-mannitol, NG+A23187, HG, or HG+BAPTA-AM. As shown in Figure 3, there was no significant threonine phosphorylation of CaMKII or serine phosphorylation of CREB after incubation in NG or NG+D-mannitol, indicating that neither NG nor hyperosmolarity induced CaMKII or CREB phosphorylation (at the sites recognized by the antibodies). In contrast, both HG and NG plus A23187 induced a significant increase in CaMKII threonine phosphorylation and CREB serine phosphorylation. In addition, the HG-induced upregulation of p-CaMKII and p-CREB was blocked by the cell permeant Ca^{2+} chelator BAPTA-AM, suggesting that HG promotes the phospho-activation of CaMKII and CREB, possibly by increasing [Ca^{2+}].

**HG activated CREB through a CaMKII-dependent pathway**

To investigate the possible role of CaMKII in CREB phosphorylation, these experiments were repeated in the presence of the CaMKII inhibitor KN93 (10 μmol/L) and the inactive analog KN92 (10 μmol/L). As shown in Figure 4, the incubation of retinal Müller cells with KN93 (but not KN92) partially reversed the HG-induced increase in CREB phosphorylation. These results indicated that CaMKII was involved in the HG-induced activation of CREB.

**CREB antisense oligonucleotides inhibits CREB expression in retinal Müller cells in NG**

As shown in Figure 5, CREB antisense oligonucleotides (CREB ASO) inhibited CREB expression in a dose-dependent manner in retinal Müller cells in NG.
Suppression of CaMKII and CREB expression decreases HG-induced HIF-1α and VEGF overexpression

To test whether CaMKII and CREB activation are required for the HG-induced upregulation of HIF-1α and VEGF expression, we examined the effects of a CaMKII specific inhibitor (KN93) and the effects of the CREB antisense oligonucleotide. We found that both treatments significantly inhibited HG-induced HIF-1α and VEGF mRNA and protein synthesis in retinal Müller cells (Figures 6A, 6B), again suggesting that the activation of CaMKII/CREB signaling may be necessary for HG-induced HIF-1α and VEGF synthesis.

Discussion

DR is a major cause of blindness in working-age individuals in developed countries[23], and aberrant angiogenesis is a central pathogenic event in the growth and progression of this disease. It has been reported that hyperglycemia-induced oxidative stress plays an important role in pathogenic retinal neovascularization and growth factor expression[24]. HIF-1 is a key oxygen sensor and mediator that regulates multiple target genes, such as VEGF, which is a key pro-angiogenic factor in DR[25, 26]. Over the past decade, almost all retinal cells have been shown to express HIF-1α and VEGF in DR. In this context, recent studies using conditional KO mice models have suggested that the Müller cell-derived HIF-1α and VEGF has a causative role in the major pathologic changes in DR[27, 28].

Increasing evidence suggests that hyperglycemia induces changes in the retinal [Ca^{2+}], dynamics[2, 4], which have been associated with distinct pathological processes, such as DR. Here, using retinal Müller cells cultured in vitro, we have demonstrated that the increased [Ca^{2+}], is associated with aberrant HG-induced expression of HIF-1α and VEGF because increased [Ca^{2+}], is an ubiquitous signal controlling gene expression[10, 29, 30]. We evaluated HG-induced HIF-1α and VEGF expression in the presence of pharmacologic inhibitors of Ca^{2+} signaling. The calcium chelators BAPTA-AM significantly reduced the production of HIF-1α and VEGF in response to HG, while the Ca^{2+} ionophore A23187 significantly increased HIF-1α and VEGF expression in the presence of normal glucose. These results suggest that elevated [Ca^{2+}], in retinal Müller cells can enhance HIF-1α expression, possibly triggering VEGF production.
Many of the cellular responses to Ca\textsuperscript{2+} are modulated by the CaMKs, among which CaMKII acts as a decoder of oscillating Ca\textsuperscript{2+} signals\cite{31}. Previous reports demonstrated that the autophosphorylation of CaMKII is highly expressed in neurons, regulating the cell cycle and transcription\cite{32}, and the effects of CaMKII were mediated by CREB phosphorylation and CREB-dependent transcription. In the present work, we show that HG-induced phosphorylation of CREB is inhibited in retinal Müller cells treated with the CaMKII inhibitor KN93 but not with its inactive analog KN92. A recent report is consistent with our findings that the CREB protein is an intracellular serine kinase that can be directly activated by CaMKII in the retina through serine-133 phosphorylation\cite{33}. However, there are very few studies investigating the downstream effects of the CaMKII-CREB pathway in retinal Müller cells. Therefore, we designed these experiments to examine the role of this pathway in the transduction of Ca\textsuperscript{2+} signals in Müller cells. Both HG and A23187 activated the CaMKII-CREB pathway and upregulated the expression of HIF-1α and VEGF in retinal Müller cells, while the chelation of calcium by BAPTA-AM partially blocked the upregulated expression of p-CaMKII/p-CREB. In addition, real-time RT-PCR and Western blot experiments indicated that both the CaMKII inhibitor KN93 and a CREB antisense oligonucleotide blocked the HG-induced upregulation of HIF-1α and VEGF. Collectively, these results strongly suggest that CaMKII-CREB may act as a key signaling pathway in the transduction of upstream Ca\textsuperscript{2+} signals into downstream gene expression after HG stimulation. Our results also support the hypothesis that CaMKII plays a role in the Ca\textsuperscript{2+}-mediated transcriptional regulation of genes through the phosphorylation of CREB to mediate a survival response in retinal ganglion cells\cite{34} because HIF-1α and VEGF could directly protect neurons from neuroexcitotoxicity. In summary, our results demonstrate that calcium contributes to HG-induced expression of the major angiogenic factors HIF-1α and VEGF in retinal Müller cells and that this response is mediated by activation of the CaMKII-CREB pathway. Calcium increase may be responsible for the hyperglycemia-induced increase in the activation of retinal Müller cells and enhanced angiogenesis in patients with diabetic retinopathy. Conversely, suppressing this pathway may be a useful strategy for novel treatments to prevent visual impairment and blindness in patients with DR.

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Author contribution
Jun LI and Shu-zhi ZHAO performed the experiments and wrote the paper; Pei-pei WANG and Song-ping YU performed the experiments and analyzed the data; and Zhi ZHENG and Xun XU designed the study.

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