

β -elemene down-regulates HIF-1 α , VEGF and iNOS in human retinal pigment epithelial cells under high glucose conditions

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Abstract

• **AIM:** To investigate the effects and mechanism of β -elemene on the expressions of hypoxia-inducible factor-1 α (HIF-1 α), vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) in human retinal pigment epithelial (RPE) cells under high glucose conditions.

• **METHODS:** ARPE-19 cell line was cultured under eight conditions: 1) low glucose (LG; 5.5 mmol/L); 2) high glucose (HG; 33 mmol/L); 3) high glucose with 20 μ g/mL β -elemene (HG+20E); 4) high glucose with 40 μ g/mL β -elemene (HG+40E); 5) high glucose with SB203590 [HG+SB203590, p38-mitogen-activated protein kinase (p38-MAPK) pathway inhibitor]; 6) high glucose with LY294002 [HG+LY294002, phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway inhibitor]; 7) high glucose with 40 μ g/mL β -elemene and SB203590 (HG+40E+SB203590); and 8) high glucose with 40 μ g/mL β -elemene and LY294002 (HG+40E+LY294002). Cells were treated in conditions 1-4 for 24 and 48h, while for 48h in conditions 5-8. Then mRNA and protein levels of HIF-1 α , VEGF and iNOS in cells were measured by real-time polymerase chain reaction (qPCR), immunofluorescence and Western blotting, respectively. Furthermore, protein levels of total p38-MAPK, phosphorylated p38-MAPK (p38-MAPK-P), total Akt and phosphorylated Akt (Akt-P) in cells of conditions 2 and 4 which treated for 48h were measured by Western blotting.

• **RESULTS:** The mRNA levels and protein levels of HIF-1 α , VEGF and iNOS in cells were significantly reduced in conditions 3-8 when compared with those in condition 2 ($P < 0.05$). These reductions were more obvious in conditions treated for 48h than in conditions treated for 24h. The protein levels of p38-

MAPK-P and Akt-P in cells of condition 4 were significantly lower than in condition 2 ($P < 0.01$).

• **CONCLUSION:** β -elemene down-regulates HIF-1 α , VEGF and iNOS in ARPE-19 cells under a high glucose condition. The inhibitory effect of β -elemene is more significant when its concentration and treatment time are increased, as well as it is combined with SB203590 or LY294002 treatment. P38-MAPK and PI3K/Akt signaling pathways may play a role in this inhibitory effect.

• **KEYWORDS:** β -elemene; retinal pigment epithelium; high glucose

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INTRODUCTION

Retinal pigment epithelium (RPE) is a main component of blood-retinal barrier (BRB) and critical for normal retinal functions. Human RPE cells secrete cytokines to regulate local immune response and play an important role for maintaining immune homeostasis in the retina. The disruption of RPE and its functions are significant features in the initiation and development of many retinal diseases^[1]. Previously, studies of diabetic retinopathy (DR) mainly focused on DR animal models and injury of vascular endothelial cells. However, it has been reported recently that other retinal cells such as RPE, Müller and ganglion cells are closely associated with the initiation and development of DR^[2]. Among these cells, RPE cells have been extensively studied because of the availability of cell lines such as ARPE-19 and D407, high success rate of culture and stable characterizations after passaging *in vitro*. It has been demonstrated that high glucose level can disrupt RPE cells and BRB, which contributes to the pathogenesis and development of DR^[3]. During RPE disruption under high glucose conditions, inducible nitric oxide synthase (iNOS) can be activated in RPE cells and excessive nitric oxide (NO) is produced to damage retinal cells^[4-5]; meanwhile, vascular

endothelial growth factor (VEGF) can be up-regulated in RPE cells with a positive correlation to glucose levels^[6]. Furthermore, the expression of VEGF is regulated by hypoxia-inducible factor-1 α (HIF-1 α)^[7]. Thus, during RPE cell injury, the levels of HIF-1 α , VEGF and iNOS in RPE cells are increased. Therefore, an agent down-regulates the levels of HIF-1 α , VEGF and iNOS in RPE cells under high glucose conditions may reduce cell injury and to be a potential strategy for DR treatment.

It has been reported that β -elemene, a natural chemical compound, inhibits VEGF and neovascularization in tumors^[8-10] and in mouse model of oxygen-induced retinopathy^[11]. Furthermore, the inhibitory effect of β -elemene on the expressions of HIF-1 α , VEGF and iNOS in the retina of a diabetic rat model had been demonstrated by our previous study^[12]. Therefore, the present study was conducted to investigate the effect and mechanism of β -elemene on the expressions of HIF-1 α , VEGF and iNOS in human RPE cells under a high glucose condition.

MATERIALS AND METHODS

Chemicals and Reagents Human RPE cell line, ARPE-19, was purchased from ATCC Co. Ltd. (AC337713, Manassas, VA, USA). β -elemene was provided by DaLian YuanDa Pharmaceutical Co., Ltd. (10 μ g/L, Dalian, Liaoning Province, China). Rabbit anti-human HIF-1 α (ab51608, Cambridge, UK), and rabbit anti-human VEGF antibodies (ab46154, Cambridge, UK) were purchased from Abcam Co., Ltd. Rabbit anti-human iNOS antibody was purchased from Santa Cruz Co., Ltd. (sc-8310, CA, USA). Rabbit anti-human β -actin and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Beijing Zhongshan Golden Bridge Co., Ltd. (BL0952, Beijing, China). SB203590 [p38-mitogen-activated protein kinase (p38-MAPK) pathway inhibitor; S1076, Houston, Texas, USA] and LY294002 [phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway inhibitor; S1105, Houston, Texas, USA] were purchased from Selleck Co. Ltd. Dimethyl sulfoxide (DMSO) was purchased from Sigma Co. Ltd. (D2650, San Francisco, CA, USA). 4',6-diamidino-2-phenylindole (DAPI; C1002, Shanghai, China), antifade mounting medium (P0126, Shanghai, China), phenylmethylsulfonyl fluoride (PMSF; ST505, Shanghai, China), goat anti-rabbit fluorescein isothiocyanate (FITC) IgG (H+L) (A0562, Shanghai, China), mouse anti-human p38-MAPK (AM065, Shanghai, China), and rabbit anti-human Akt antibodies (AA311, Shanghai, China) were purchased from Beyotime Co., Ltd. Cell culture plates (3516, New York, NY, USA) and bottles (430639, New York, NY, USA) were purchased from Corning Co. Ltd.

Cell Culture and Treatment ARPE-19 cells were cultured in Dulbecco's modified eagle medium (DMEM, SH30021.01 or

SH30022.01B, Hyclone, Logan, Utah, USA) with 10% fetal bovine serum (SH30088.03, Hyclone, Logan, Utah, USA). The cells were digested by trypsin (25200-056, Gibco, Grand Island, NY, USA) in the exponential growth phase. Then single cell suspension (1×10^6) was made in low glucose (5.5 mmol/L) DMEM with 10% fetal bovine serum and seeded on a six-wells culture plate. When cells attached to the plate as a monolayer and approached 80%-90% confluence, culture medium was changed to DMEM with 1% fetal bovine serum for overnight serum starvation. Then cells were cultured again in DMEM with 10% fetal bovine serum and treated under eight conditions: 1) low glucose (LG; 5.5 mmol/L); 2) high glucose (HG; 33 mmol/L); 3) high glucose with 20 μ g/mL β -elemene (HG+20E); 4) high glucose with 40 μ g/mL β -elemene (HG+40E); 5) high glucose with SB203590 (HG+SB203590); 6) high glucose with LY294002 (HG+LY294002); 7) high glucose with 40 μ g/mL β -elemene and SB203590 (HG+40E+SB203590); and 8) high glucose with 40 μ g/mL β -elemene and LY294002 (HG+40E+LY294002). Cells were cultured in conditions 1-4 for 24 and 48h, while for 48h in conditions 5-8.

Immunofluorescence Staining After the cells were treated under eight conditions, the slides of cells were put in a 35 or 60 mm culture dish and washed by phosphate buffer saline (PBS) for three times. Then the cells were fixed in cold 4% paraformaldehyde for 20min, permeabilized by 0.4% Triton X-100 for 20min, blocked in 1% bovine serum albumin (BSA) for 30min, incubated with primary antibody (1:500 HIF-1 α antibody, 1:200 VEGF antibody and 1:50 iNOS antibody) in a humidified chamber at 4°C overnight, treated with second antibody (1:100) at room temperature for 1h in dark, and stained by DAPI (5 μ g/mL) for 2min. Between each procedure, the cells were briefly rinsed in PBS for three times. Finally, the slides of cells were mounted by antifade mounting medium and imaged under a fluorescence microscope (BX43, Olympus, Tokyo, Japan).

Quantitative Real-time Polymerase Chain Reaction Total RNA was extracted using Trizol method (Life Technologies, Carlsbad, USA). Then OD260 and OD280 were measured for 1 μ L RNA using an ultraviolet spectrophotometer (SP-1920, SPECTRUM, Shanghai, China). The ratio of OD260/280 was calculated and range 1.8-2.0 was considered as high quality RNA used for reverse transcription. Reverse transcription was performed at 37°C for 15min and heat inactivation of reverse transcriptase was conducted at 85°C for 5s. Then quantitative real-time polymerase chain reaction (qPCR) was performed using 25 μ L reaction volume system and the conditions were set as follows: 95°C for 10min (initial-denaturation), 95°C for 15s (denaturation), and 55°C for 30s (annealing and extension); 40 cycles. β -actin was set as the internal control.

The sequences of HIF-1 α , VEGF, iNOS, and β -actin primers are shown in Table 1.

Western Blot The Bradford method was used to measure the protein concentration with reagents purchased from the Bio-Rad (Hercules, CA, USA). Then sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 90 V for 30min followed by 120 V for 1h. The separated proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane using 70 V for 60min. The membrane containing the protein bands was serially incubated with: 1) non-fat dry milk at room temperature for 2h; 2) primary antibody [diluted in 0.05% Tris buffered saline with Tween-20 (TBST) as 1:1000, 4°C, overnight]; 3) second antibody (diluted in 0.05% TBST as 1:5000, room temperature, 1h). Between these three procedures, the membrane was rinsed in 0.05% TBST for three times and each for 10min. Finally, electrochemiluminescence (ECL, Amersham-Pharmacia Biotech, Beijing, China) was used to make the protein bands visible and grayscale values of the bands were analyzed using Image J2x software (Rawak Software Inc., Stuttgart, Germany). The grayscale value of β -actin was set as the internal control.

Statistical Analysis SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The variables data are presented as mean \pm standard deviation (SD). The Student's *t*-test was used for normally distributed data. For data do not meet normal distribution, rank sum test was used for the analysis. Chi-square test was used for comparison of ratios. $P < 0.05$ was considered as statistically significant, while $P < 0.01$ was considered as there was significant statistical significance.

RESULTS

Immunofluorescence Analysis The results are shown in Figure 1. For LG condition, there were small amounts of HIF-1 α and iNOS expressed in the cytoplasm and nucleus, and small amount of VEGF expressed in the cytoplasm. For HG condition, the intense of fluorescence indicates HIF-1 α , VEGF and iNOS increased dramatically. In conditions 3 and 4, after 24h treatment of β -elemene, the intense of fluorescence in the cytoplasm reduced when compared with HG condition. This fluorescence reduction is more obvious when β -elemene concentration was 40 μ g/mL and treatment time was 48h.

Quantitative Real-time Polymerase Chain Reaction The mRNA levels of HIF-1 α , VEGF and iNOS as well as the comparison results between groups are shown in Figure 2. The mRNA level of HIF-1 α is significantly higher in HG condition than in LG condition ($P = 0.001$ for 24h, $P = 0.002$ for 48h). There was no statistical significance between HIF-1 α mRNA in HG+20E (24h) and in HG condition ($P = 0.182$) while the inhibition is significant in HG+20E (48h; $P = 0.041$).

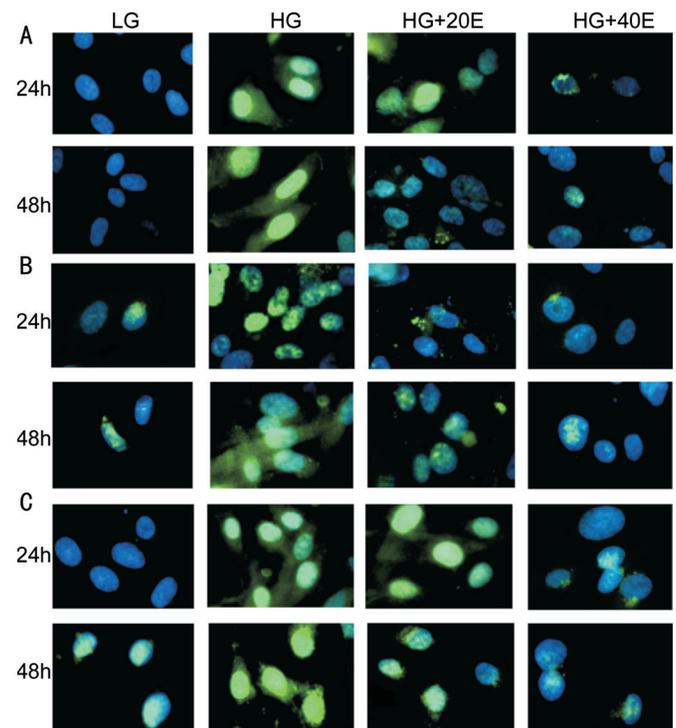


Figure 1 Immunofluorescence results of the expression levels of HIF-1 α , VEGF, and iNOS in ARPE-19 cells ($\times 40$) A: HIF-1 α ; B: VEGF; C: iNOS. For LG condition, there are small amount of HIF-1 α and iNOS expressed in the cytoplasm and nucleus, and small amount of VEGF expressed in the cytoplasm. In HG condition, more intense immunofluorescence (green) is observed in the cytoplasm and co-localized with DAPI-stained nuclei (blue). Less immunofluorescence (green) is observed in HG+20E (24 and 48h) and HG+40E (24 and 48h) conditions when compared with HG conditions. It is more obvious in HG+20E (48h) and HG+40E (48h) conditions.

Table 1 The sequences of HIF-1 α , VEGF, iNOS and β -actin primers

Name	Sequence (5'-3')	Size
VEGF F	TTCTGAGTTGCCAGGAGAC	173
VEGF R	TGGTTTCAATGGTGTGAGGA	
HIF-1 α F	TGCAACATGGAAGGTATTGC	104
HIF-1 α R	CCAAGCAGGTCATAGGTGGT	
iNOS F	GAGGCAAACAGCACATTCAG	135
iNOS R	CAGGAGAGTTCCACCAGGAT	
β -actin F	GTGATCTCCTTCTGCATCCTGT	132
β -actin R	CCACGAAACTACCTTCAACTCC	

HIF-1 α : Hypoxia-inducible factor-1 α ; VEGF: Vascular endothelial growth factor; iNOS: Inducible nitric oxide synthase; F: Forward; R: Reverse. Size unit: Bases.

The inhibition of HIF-1 α mRNA was significant in HG+40E (24h) and HG+40E (48h) conditions ($P = 0.045$ for 24h, $P = 0.034$ for 48h). The mRNA level of VEGF was significantly higher in HG condition than in LG condition ($P = 0.002$ for

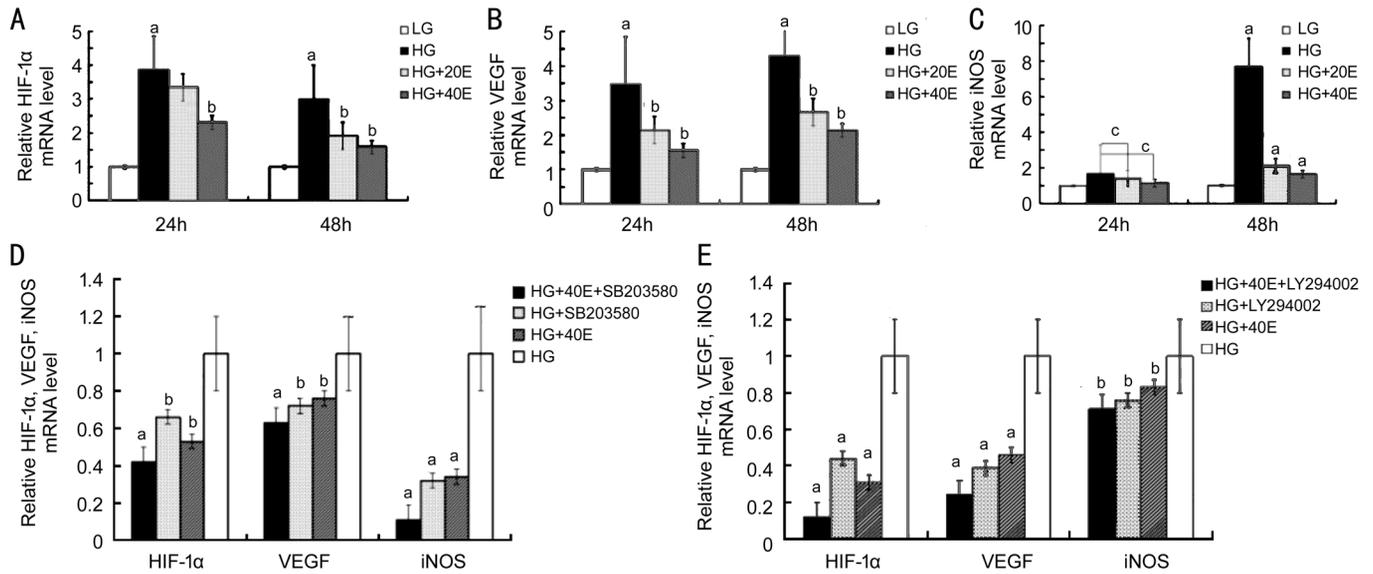


Figure 2 Real-time PCR results of mRNA expressions of HIF-1 α , VEGF and iNOS in ARPE-19 cells A: mRNA level of HIF-1 α after β -elemene treatment for 24 and 48h; B: mRNA level of VEGF after β -elemene treatment for 24 and 48h; C: mRNA level of iNOS after β -elemene treatment for 24 and 48h; D: mRNA levels of HIF-1 α , VEGF and iNOS after 40 μ g/mL β -elemene and SB203580 treatments for 48h; E: mRNA levels of HIF-1 α , VEGF and iNOS after 40 μ g/mL β -elemene and LY294002 treatments for 48h. ^a P <0.01, ^b P <0.05, ^c P >0.05.

24h, $P=0.001$ for 48h). The inhibition of VEGF mRNA was significant in HG+20E ($P=0.043$ for 24h, $P=0.041$ for 48h) and HG+40E ($P=0.035$ for 24h, $P=0.036$ for 48h) conditions. The mRNA level of iNOS was significantly higher in HG (48h) condition than in LG condition ($P=0.001$). The inhibition of iNOS mRNA was not significant in HG+20E (24h; $P=0.605$) and HG+40E (24h; $P=0.588$) conditions while the inhibition was significant in HG+20E (48h; $P=0.028$) and HG+40E (48h; $P=0.028$) conditions. The mRNA levels of HIF-1 α and VEGF were reduced significantly in HG+40E ($P=0.018$ for HIF-1 α , $P=0.046$ for VEGF) and HG+SB203580 conditions when compared with HG condition ($P=0.038$ for HIF-1 α , $P=0.040$ for VEGF), and more significant in HG+40E+SB203580 condition ($P=0.0096$ for HIF-1 α , $P=0.040$ for VEGF). The mRNA levels of iNOS were reduced very significantly in HG+40E ($P=0.007$), HG+SB203580 ($P=0.005$) and HG+40E+SB203580 ($P=0.003$) conditions when compared with HG condition. The mRNA levels of iNOS in HG+40E ($P=0.040$), HG+LY294002 ($P=0.034$) and HG+40E+LY294002 ($P=0.034$) conditions were reduced significantly when compared with HG condition (P <0.05). The mRNA levels of HIF-1 α and VEGF in HG+40E ($P=0.004$ for HIF-1 α ; $P=0.006$ for VEGF), HG+LY294002 ($P=0.006$ for HIF-1 α ; $P=0.005$ for VEGF) and HG+40E+LY294002 ($P=0.003$ for HIF-1 α , $P=0.004$ for VEGF) conditions were reduced more significantly (P <0.01).

Western Blot The protein levels of HIF-1 α , VEGF and iNOS as well as the comparison results between groups are shown in Figure 3. The protein levels of HIF-1 α , VEGF and iNOS were significantly higher in HG condition than in LG condition

(P <0.05, P <0.01 and P <0.01, respectively). The inhibition of HIF-1 α , VEGF proteins was significant in HG+20E (24h) condition (P <0.05) and more significant in HG+40E (24h) condition (P <0.01) when compared with HG condition. iNOS protein was significantly reduced in HG+20E and HG+40E conditions (P <0.01) when compared with HG condition.

The protein levels of HIF-1 α , VEGF and iNOS were reduced significantly in HG+SB203580, HG+LY294002, HG+40E+SB203580, and HG+40E+LY294002 conditions when compared with HG condition (P <0.01; Figure 4A, 4B). The inhibitory effect is more obvious in HG+40E+SB203580 and HG+40E+LY294002 conditions.

The protein levels of total p38-MAPK, p38-MAPK-P, total Akt and Akt-P in HG and HG+40E conditions which treated for 48h were compared. p38-MAPK-P and Akt-P were reduced significantly in HG+40E condition when compared with HG condition (P <0.01; Figure 4C).

DISCUSSION

High glucose damages RPE cells and affects the function of BRB, plays an important role for the initiation and development of DR. Under high glucose conditions, alteration of cytokines in human RPE cells initiates a series of signaling transfer in cells, and induces cell damage and neovascularization^[13-15]. In the present study, in order to observe the effect of β -elemene on RPE cells under high glucose conditions, we focus on the alterations of HIF-1 α , VEGF and iNOS in RPE cells. Because it has been reported that HIF-1 α , VEGF and iNOS up-regulated in RPE cells under high glucose conditions^[16-17]. The up-regulation of HIF-1 α can further induce the increased level of VEGF^[18-21]. VEGF is a key factor of various neovascular

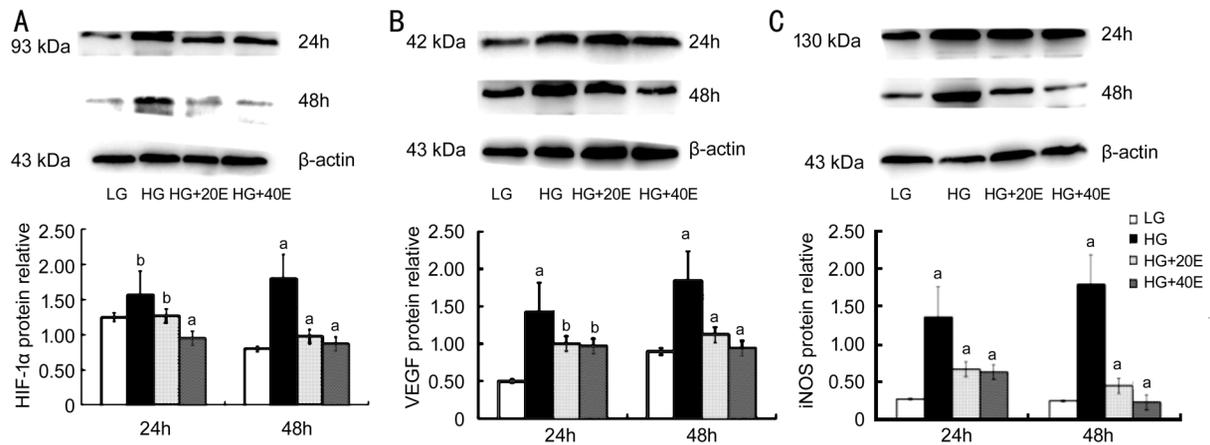


Figure 3 Western blot results of HIF-1 α , VEGF and iNOS in ARPE-19 cells Protein levels of HIF-1 α , VEGF and iNOS are significantly higher in HG condition than in LG condition. A: Protein level of HIF-1 α after β -elemene treatment for 24 and 48h. The inhibition of HIF-1 α protein is significant in HG+20E (24h) and more significant in HG+40E (24h) condition when compared with HG condition. B: Protein level of VEGF after β -elemene treatment for 24 and 48h. The inhibition of VEGF protein is significant in HG+20E (24h) and more significant in HG+40E (24h) condition when compared with HG condition. C: Protein level of iNOS after β -elemene treatment for 24 and 48h. The inhibition of iNOS protein is more significant in HG+20E and HG+40E conditions) when compared with HG condition. ^a P <0.01, ^b P <0.05.

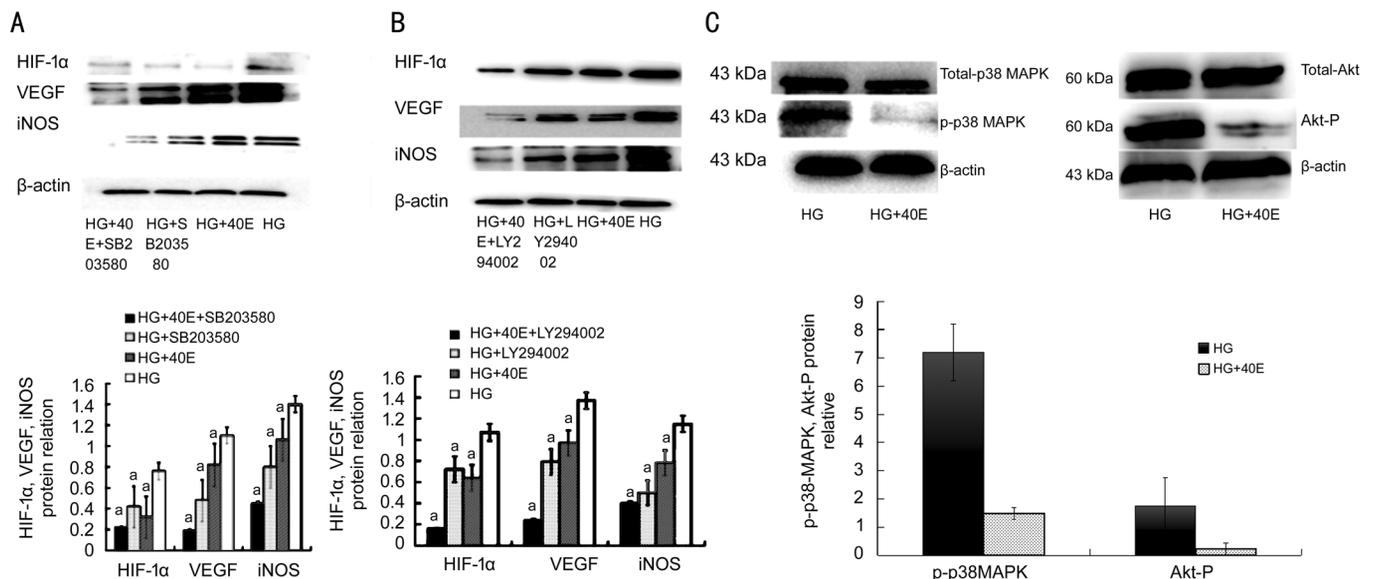


Figure 4 Western blot results of protein levels of HIF-1, VEGF, iNOS, total p38-MAPK, p38-MAPK-P, total Akt and Akt-P in ARPE-19 cells A: Protein levels of HIF-1 α , VEGF and iNOS after 40 μ g/mL β -elemene and SB203580 treatments for 48h. Protein levels of HIF-1 α , VEGF and iNOS are reduced significantly in HG+SB203580 and HG+40E+SB203580 conditions when compared with HG condition. B: Protein levels of HIF-1 α , VEGF and iNOS after 40 μ g/mL β -elemene and LY294002 treatments for 48h. Protein levels of HIF-1 α , VEGF and iNOS are reduced significantly in HG+LY294002 and HG+40E+LY294002 conditions when compared with HG condition. C: Protein levels of total p38-MAPK and p38-MAPK-P, total Akt and Akt-P in HG and HG+40E groups. p38-MAPK-P or Akt-P is reduced significantly in HG+40E condition when compared with HG condition. ^a P <0.01.

retinopathy. Pathogenic factors promote the neovascularization through VEGF and VEGF can up-regulate the expressions of other cytokines and growth factors. Therefore, it has been postulated that inhibition of HIF-1 α and finally down-regulates VEGF could be a strategy of treating ischemic retinopathy^[22-26]. The up-regulation of iNOS under high glucose conditions induces excessive NO and damage the vascular endothelial cells. NO is a typical free radical. The normal function of NO

is to dilate blood vessels and maintain the blood supply to tissues. However, extensive NO reacts with superoxide anion and produces peroxynitrite (ONOO⁻), which is a very strong oxidant. It can disrupt the function of vascular endothelial cells directly by lipid peroxidation, and up-regulate the expressions of adhesion molecules and inflammatory factors to induce the chronic complications of diabetes mellitus (DM)^[27]. In addition, extensive peroxynitrite (ONOO⁻) increases the level

of nitrotyrosine and reduces the biological activity of NO, and finally causes DNA injury. Nitrite measured by Griess test can reflect the relative amount of NO in cells. However, Griess test was not applied in the present study because of the volatility of the measurements from the test. The expression of NO was reflected by measuring iNOS level in cells instead.

It has been reported that β -elemene has effect of anti-neovascularization in tumors. It can down-regulate the expressions of VEGF and its receptors in lung adenocarcinoma, laryngeal squamous cell carcinoma, gastric cancer and melanoma to inhibit neovascularization, tumor growth and metastasis^[28-30]. Therefore, β -elemene is a natural inhibitor for neovascularization. Furthermore, the inhibitory effect of β -elemene on the expressions of HIF-1 α , VEGF and iNOS in the retina of a diabetic rat model was revealed in our previous study and indicates the potential value of β -elemene on proliferative retinopathy treatment^[12].

Studies on tumors have revealed that the effects of β -elemene are associated with p38-MAPK and PI3K/Akt pathways. P38 signaling pathway is an important component of MAPK family and can be activated by stresses include ultraviolet rays, radiation, heat shock, proinflammatory factors, and specific antigen, *etc.* P38-MAPK has important effects in apoptosis, cytokine production, transcriptional regulation and ischemia-reperfusion injury^[31-35]. Because p38-MAPK signaling plays an important role in retinal inflammation induced by DM, it is a key to inhibit p38-MAPK in early DR and other DM complications^[36].

P38-MAPK signaling is also associated with the regulation of VEGF. The regulation of VEGF mainly includes three pathways: 1) through p42/44, such as the regulation of insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF) and proto-oncogenes; 2) through activating protein 2 (AP-2), when HIF-1 induced by ischemic tissues; and 3) through JNK/p38, to enhance the effect of VEGF by increasing stability of VEGF mRNA. In DM, AP-2 and JNK/p38 pathways are involved in the process of the disorder. Ischemia in DM can up-regulate the expression and activity of HIF-1, which is a regulatory gene at the upstream of VEGF; on the other hand, a RNA protein complex can be formed at the adenylate-uridylylate-rich element of 3' untranslated region (UTR) of VEGF mRNA to regulate the stability of VEGF mRNA and promotes the expression of VEGF protein. Thus, a feedback system is generated between VEGF and MAPK signaling, and the initiation and development of DR are accelerated^[37-38].

VEGF can be produced by RPE cells, and the endothelial cells and pericytes of retinal vessels. Among them, RPE cells have the most significant role in producing VEGF^[39]. It has been reported that the activation of p38-MAPK pathway in DM rats is through the upregulation of p38-MAPK-P^[40].

P38-MAPK inhibitors have been studied and developed extensively^[38-39]. Among them, SB203580 is the selective inhibitor of p38-MAPK and is the one applied most extensively. It inhibits p38-MAPK and the following phosphorylation of heat shock protein 27 (Hsp27) to inhibit the activation of MAPK-activated protein kinase 2 (MAPKAPK2). The inhibition effect of SB203580 is through the phosphorylation after it binds to the position where p38-MAPK binds to ATP.

Furthermore, it has been reported that PI3K/Akt pathway is associated with the formation of fibrovascular membranes in proliferative DR and age-related macular degeneration. The PI3K/Akt pathway is considered as an important potential target for treating neovascular retinopathy^[41-43]. LY294002 is a protein kinase inhibitor and has been applied extensively in the research for PI3K signaling pathway. LY294002 can penetrate cells and specifically inhibits PI3K, PI3K/Akt signaling pathway and Akt phosphorylation. Akt plays a key role in the growth and apoptosis of cells and can be activated by insulin and various growth and survival factors^[44-46].

In the present study, expression of proteins and mRNAs of HIF-1 α , VEGF and iNOS in RPE cells cultured under a high glucose condition were measured to test if the treatment of β -elemene reduce these expressions and have potential protection on RPE cells. In our preliminary test, cell proliferation assay (MTS) was applied to observe the viability of cultured human RPE cells in various culture conditions. The time points of β -elemene treatment were set at 12, 24, 36 and 48h, and the treatment concentrations of β -elemene were selected as 20, 40, 80 and 160 μ g/mL. The results showed that the cell protection effect of β -elemene started from 20 μ g/mL and 24h, while 80 and 160 μ g/mL β -elemene did not improve cell viability when compared with HG condition. Therefore, β -elemene concentrations finally used in the present study were 20 and 40 μ g/mL, and treatment times were 24 and 48h. The results showed that the effects of β -elemene was time and dose dependent in these conditions. The inhibition of levels of HIF-1 α , VEGF and iNOS was enhanced when the time and concentration increased. P38-MAPK inhibitor SB203580 and PI3K/Akt inhibitor LY294002 were applied in the present study, respectively, or combined with β -elemene, to test the possible pathways involved in β -elemene effect. The results showed that they can inhibit the expressions of HIF-1 α , VEGF and iNOS. The inhibition was enhanced by the combination of β -elemene. Therefore, we postulated that the inhibition effect of β -elemene showed in the present study is probably through p38-MAPK and PI3K/Akt signaling pathways. However, there are some limitations in the present study. Since VEGF is a secretory protein, further ELISA analysis is warranted to determine its level in the supernatant of culture cells. Furthermore, the molecular mechanism of down-regulating

HIF-1 α , VEGF and iNOS by β -elemene and the effect of β -elemene on vascular endothelial cells are not clear and warrant further studies.

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Conflicts of Interest: Zhou Y, None; Chen J, None; Li LH, None; Chen L, None.

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