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ORIGINAL PAPER

Critical effect of VEGF in the process of endothelial cell apoptosis induced by high glucose

Zhonghan Yang · Xuehua Mo · Qing Gong · Qiuhui Pan · Xia Yang · Weibin Cai · Chaoyang Li · Jian-xing Ma · Yunshao He · Guoquan Gao

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Abstract The underlying molecular mechanism whereby hyperglycemia causes endothelial cell apoptosis is not well understood. This study aims to elucidate the role of survival factor VEGF involved in the apoptosis of endothelial cells induced by elevated glucose. The present study confirmed that high concentration of glucose (25 mmol/l) significantly increased the apoptotic cell number in cultured primary human umbilical vein endothelial cells (HUVEC). Up-regulation of Bax/Bcl-2 ratio and activation of caspase-3 induced by high glucose suggested that mitochondria apoptosis pathway was involved. High glucose significantly reduced VEGF expression in HUVEC both at mRNA and protein levels. p42/44 MAPK phosphorylation was transitory attenuated when exposed to high glucose and preceded VEGF reduction, thus suggesting down-regulation of VEGF through inhibition of p42/44 MAPK. Addition of VEGF prevented HUVEC apoptosis from high glucose exposure. Moreover, elevated reactive oxygen species (ROS) generation, calcium overload, Bax/Bcl-2 ratio, caspase-3 activation in HUVEC induced by high glucose were reversed by pre-challenge with VEGF. This may represent a mechanism for the anti-apoptotic effect of VEGF. These results suggest that down-regulation of VEGF plays a critical role in apoptosis of endothelial cells induced by high glucose and restoration of VEGF might have benefits in the early stage of diabetic endothelial dysfunction.

Keywords Hyperglycemia · Endothelial cells · Apoptosis · VEGF · Caspase-3 · Bax

Abbreviations

Glc	Glucose
HUVEC	Human umbilical vein endothelial cells
MAPK	Mitogen-activated protein kinase
ROS	Reactive oxygen species
VEGF	Vascular endothelial growth factor

Zhonghan Yang, Xuehua Mo, and Qing Gong have contributed equally to this study.

Z. Yang · Q. Gong · X. Yang · W. Cai · G. Gao (✉)
Department of Biochemistry, Zhongshan Medical School, Sun Yat-sen University, 74 Zhongshan 2nd Road, Guangzhou 510080, China
e-mail: gaogq@mail.sysu.edu.cn; biolin@yahoo.cn

X. Mo · Y. He
Department of Anatomy, Zhongshan Medical School, Sun Yat-sen University, Guangzhou 510080, China

Q. Pan
Medical Research Center of the Second Affiliated Hospital, Sun Yat-sen University, Guangzhou 510120, China

W. Cai
China Key Laboratory of Tropical Disease Control, Ministry of Education, Sun Yat-sen University, Guangzhou 510080, China

C. Li
State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, China

J.-x. Ma
Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City 73104, OK, USA

G. Gao
Guangdong Province Key Laboratory of Functional Molecules in Oceanic Microorganism, Office of Education, Sun Yat-sen University, Guangzhou 510080, China

Introduction

Complications of diabetes such as retinopathy of prematurity (ROP) and diabetic nephropathy are characterized with the premature development of microvascular and macrovascular disease, and endothelial cell dysfunction is an early marker of such complications [1, 2]. Hyperglycemia is an independent risk factor for the development of cardiovascular disease. However, the mechanism of hyperglycemia-related tissue damage and clinical complications still remain unclear. Previous in vitro studies have confirmed that exposure of human endothelial cells to high glucose induces significant cell death via apoptosis [3, 4]. Documents reported that apoptosis is particularly prominent in models of hyperglycemia injury, affecting a significant proportion of vascular endothelium in the tissue damage [5, 6].

Several mechanisms such as disturbed metabolic pathways, reactive oxygen species (ROS) and altered release of growth factors may account for the apoptosis induced by elevated glucose [7–10]. Previous studies have shown that high glucose phosphorylates p38 MAPK and alters signal transduction pathways in vascular cells, phosphorylation of p38 MAPK downstream of Bax-caspase-3 pathway leads to endothelial cell apoptosis induced by high glucose [6]. Increased reactive oxygen species (ROS) and intracellular Ca^{2+} elevation were involved in apoptosis induced by high glucose [8, 9, 11–14]. In addition, intracellular survival factors expression changes also can mediate apoptosis. Specifically, VEGF, as a critical survival factor for endothelial cells, have been proposed to inhibited apoptosis [15]. VEGF can exert a vascular protective effect by activating anti-apoptotic kinase and maintaining survival signals in endothelial cells [16, 17]. It was reported that VEGF suppresses the apoptosis of sinusoidal endothelial cells caused by cold preservation [18], human dermal microvascular endothelial cells and human umbilical vein endothelial cells induced by starvation and ceramide [15], and bovine aortic endothelial cells induced by TNF-α and H_2O_2 [17]. These results suggested that VEGF is an important endothelial cell survival factor and may also arrest endothelial cell apoptosis induced by other conditions such as exposure to high glucose. However, the expression changes and possible protective effect of VEGF on apoptosis in endothelial cells induced by high glucose have not been well explored.

The present studies examined the direct effects of constant high concentration of glucose on VEGF expression and the underlying mechanism for protective effect of VEGF on apoptosis of endothelial cells induced by high glucose. This study aims to elucidate the pivotal anti-apoptotic role of survival factor VEGF in endothelial cells underneath the circumstance of elevated glucose.

Materials and methods

Culturing, identification and counting of HUVEC

HUVEC were isolated from umbilical vein cords of normal pregnancies following a protocol described previously [19] with some modifications. These cells were cultured on flasks coated with 0.2% gelatin in medium SFM supplemented with 15% fetal bovine serum (Gibco BRL, Gaithersburg, MD), 15 mg/l ECGS (Upstate, N.Y. USA) in an atmosphere of 5% CO_2 at 37°C. The medium was changed every 3–4 days until the cells reached confluence. Rabbit anti-human von Willebrand factor polyclonal first antibody (Gene Tech, Shanghai, China) and FITC-labelled anti-rabbit immunoglobulin/FITC secondary antibody (Dako, Glostrup, Denmark) were used to identify HUVEC by Immunocytochemistry method. The number of live cells was counted by using blood counting instrument after 0.4% trypan blue staining. To maintain uniform condition, all experiments were carried out between cell passages 4 and 6.

Treatment of HUVEC with high glucose

The cells were seeded onto gelatin-coated tissue culture plates or dishes and incubated with the medium containing either 25 mmol/l glucose or 5 mmol/l glucose plus 20 mmol/l mannitol (to maintain equal osmolarity of all cultures). For effect of VEGF on endothelial cell, HUVEC were grown to ~80% confluence in 100-mm cell culture dishes, and the medium was replaced with SFM endothelial cell medium containing 20 ng/ml bFGF, 20 ng/ml EGF and 5% FBS in the presence or absence of 20 ng/ml VEGF. The cells were exposed to different concentration of glucose for indicated time and harvested for flow cytometry and Western blot analysis.

Analysis of apoptosis by Hoechst 33258 and Annexin V/PI staining

DNA chromatin morphology was assessed using Hoechst staining. HUVEC were incubated with 25 mmol/l glucose and/or other predetermined reagents for 3 days. Cells were washed by PBS and labeled with 5 μg/ml of Hoechst 33258 for 10 min, and the cells were examined by fluorescence microscopy. Annexin V/PI staining was as described previously [20]. Briefly, HUVEC were plated at a density of 10^5 cells per well in 6-well plates, and then exposed to glucose (25 mmol/l) and VEGF (20 ng/ml) for 3 days and harvested for Annexin V and propidium iodide (PI) staining using the Annexin V-FITC Apoptosis Detection Kit (Sigma, St. Louis, Mo., USA), and the cells were subsequently counted by flow cytometry (Coulter, Hialeah, FL). Colchicine (Sigma), which is known to induce apoptosis by

disrupting microtubules and preventing its polymerization, was used as a positive control.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from HUVEC according to the manufacture's instructions for Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was converted to first strand cDNA and then subjected to semiquantitative PCR analysis for VEGF. The identity of PCR products was examined by 1.5% agarose gel electrophoresis. β-actin gene was used as internal control. Intensity of bands was semiquantified by GeneGenius densitometry and analyzed by GeneTool program (Gene Ltd. Hong Kong, China). The ratio of the band intensity between internal control and gene of interest was calculated. The nucleotide sequences of the specific primers used are summarized in Table 1.

Analysis of protein levels by Western blotting

HUVEC were exposed to the various experimental conditions for predetermined periods, with osmotic controls. Then the cells were harvested and lysed for total protein extraction. Protein concentration was determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein (100 μg) from the cell lysates were subjected to Western blot analysis for the VEGF, Bax, Bcl-2, caspase-3, total and phosphorylated (phosphor-) p42/44 MAPK using an ECL detection kit. The same membrane was stripped and re-blotted with an antibody specific to β-actin or COX IV. VEGF, Bax and Bcl-2 concentrations were normalized by β-actin, and COX IV was used as a mitochondrial loading control.

Caspase-3 activity assay and ELISA of VEGF

Caspase-3 activity was determined by a colorimetric assay based on the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-*p*NA) into a yellow formazan product (*p*-nitroanilide (*p*NA)). An increase in absorbance at 405 nm was used to quantify the activation of caspase-3 activity. After 48 h exposure, the supernatants of medium and HUVEC were collected respectively. The cells were rinsed with cold PBS, and then lysed by lysis buffer (100 μl/2 $\times$ 10^6 cells) for 15 mins on ice. Cell lysates were centrifuged at 18,000*g* for 10 mins at 4°C. Caspase-3 activity in the supernatant was assayed using the kit (Beyotime, China). The caspase-3 activity was expressed as percentage of enzyme activity compared to control. The concentration of VEGF in the culture medium without dilution was measured using an ELISA kit (JINGMEI Biotech, China) against human VEGF. The experiment was carried out in triplicates.

Table 1 Primers employed in RT-PCR analysis

Gene	Nucleotide sequence	Amplicon (bp)
VEGF	Sense-gAgggCAgAATCATCACgAA	401
	Antisense-gggAACgCTCCAggACTTAT	
β-actin	Sense-TCATCACCATTggCAATgAg	155
	Antisense-CACTgTgTTggCgTACAggT	

Detection of intracellular hydrogen peroxide production

Intracellular hydrogen peroxide (H_2O_2) production was measured by Fenton reaction which is the common chemical reaction producing hydroxyl free radical (·OH). The level of H_2O_2 is in direct proportion to the amount of ·OH produced by Fenton reaction. Cells (2×10^5/ml) were treated with high concentration of glucose (25 mmol/l) for predetermined periods and reactive oxygen species (ROS) was measured with Hydroxyl Free Radical Detection Kit (Nanjing Jiancheng Bioengineering Company, China) by Fenton reaction. Gress reagent was used to initiate color reaction and the absorbance was read at 550 nm on a spectrophotometer.

Determination of $[Ca^{2+}]_i$ change in cultured HUVEC

The change of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the cultured HUVEC was measured using fluorescence probes fluo-3 with acetoxymethyl ester. The dyes were loaded into the cells by adding the 5 μmol/l fluo-3 AM from 1 mmol/l stock in DMSO to MOPS buffer and incubated for 30 min at 37°C in the dark. After loading, the cells were washed three times with dye-free MOPS-buffer and measured the fluorescence density. Florescence images of the intracellular Ca^{2+} localization were obtained using a laser-scanned confocal microscope (Olymbus, FV500) equipped with an argon ion laser and a fluorescein (488 nm) filter cartridge. The fluorescein filter was used to excite the fluo-3. Thus, when excited at 488 nm, the fluo-3 exhibits an increase in green fluorescence (525 nm) on Ca^{2+} binding.

Statistical analysis

Comparisons were performed using Student's *t*-test and analysis of variance for repeat measurements. All data from three separate experiments are given as mean ± SD.

Results

Effect of high glucose on the proliferation and apoptosis of HUVEC

The purity of the culture was examined by anti-vWF/FITC staining which showed positive staining (Fig. 1b), indicating their identity as endothelial cells. After random counting five microscope fields for three independent experiments, the average percent of vWF positive staining cells is about 90% which reflects the purity of HUVEC. HUVEC which were cultured for 6 days in high concentration of glucose had a significant decrease in viability as shown by trypan blue exclusion in comparison with those cultured in low glucose of 5 mmol/l glucose [$(6.0 \pm 1.0) \times 10^4$ cells/well vs. $(10.0 \pm 0.9) \times 10^4$ cells/well, $P < 0.05$] (Fig. 1d). No significant decrease in cell number was observed in the presence of 15 mmol/l glucose [$(9.6 \pm 1.2) \times 10^4$ cells/well, $P < 1.0$]. The effect of high glucose was not attributable to hyperosmolarity of the medium since 20 mmol/l mannitol was added to the low glucose medium, which was identical to the medium of osmotic pressure of 25 mmol/l glucose.

To determine whether the cytotoxicity of high glucose on HUVEC is due to induction of cell death, cell apoptosis was analyzed by Hoechst 33258 and Annexin V/PI staining. As shown in Fig. 2, the proportion of apoptotic cells labeled with Hoechst 33258 was increased from $4.3 \pm 0.69\%$ in 5 mmol/l glucose-treated HUVEC to $15.1 \pm 4.4\%$ in 25 mmol/l glucose-treated HUVEC. As shown in Fig. 3a–d, the proportion of apoptotic cells (including early and late phase) labeled with Annexin V/PI was increased from $3.3 \pm 0.44\%$ in 5 mmol/l glucose-treated HUVEC (Fig. 3a) to $14.1 \pm 3.3\%$ in the high glucose-treated HUVEC (Fig. 3d), suggesting a more than four-fold increase in apoptotic cells. The effect of high glucose was not also attributable to hyperosmolarity (Fig. 3b). These results suggested that high glucose induces endothelial cell death via apoptosis.

High glucose up-regulates Bax/Bcl-2 ratio and activates caspase-3 in HUVEC

To further examine the molecule mechanism of the apoptosis pathway, we examined the apoptosis-related protein Bax/Bcl-2 and the cleavage of caspase-3 induced by high glucose. High glucose treatment obviously increased Bax protein expression analyzed by Western blotting (Fig. 4a), whereas no obviously change in Bcl-2 protein was observed (Fig. 4b). The ratio of Bax/Bcl-2 was increased two-fold by high glucose in total homogenate (Fig. 4c). The ratio of Bax/Bcl-2 in mitochondrial was also examined as normalized by mitochondrial marker COX IV. The Bax protein amount which moves to mitochondria is increased

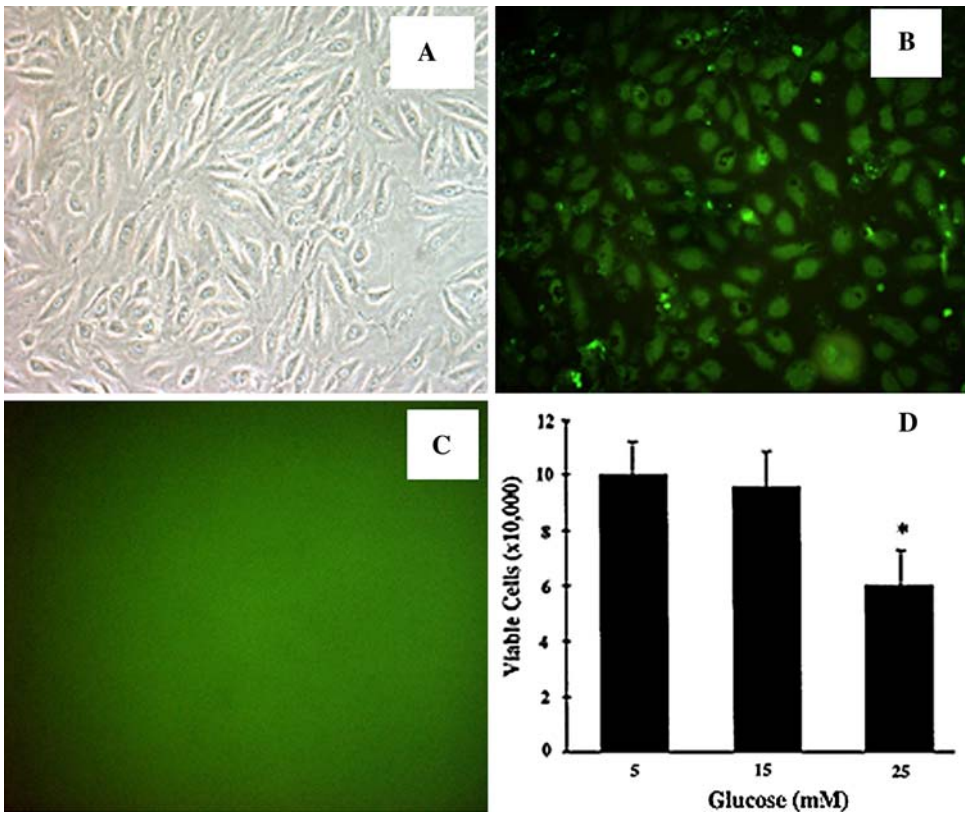

Fig. 1 Identification of cultured HUVEC and effect of high glucose on the endothelial cells. (**a**) Confluent field by light microscopy (mag. 200×). (**b**) Staining identification of HUVEC by anti-vWF/FITC (mag. 200×). (**c**) Control (without anti-vWF and only with FITC). (**d**) Effects of high concentration of glucose on the proliferation of HUVEC through trypan blue staining. * $P < 0.05$ vs. 5 mmol/l of glucose group

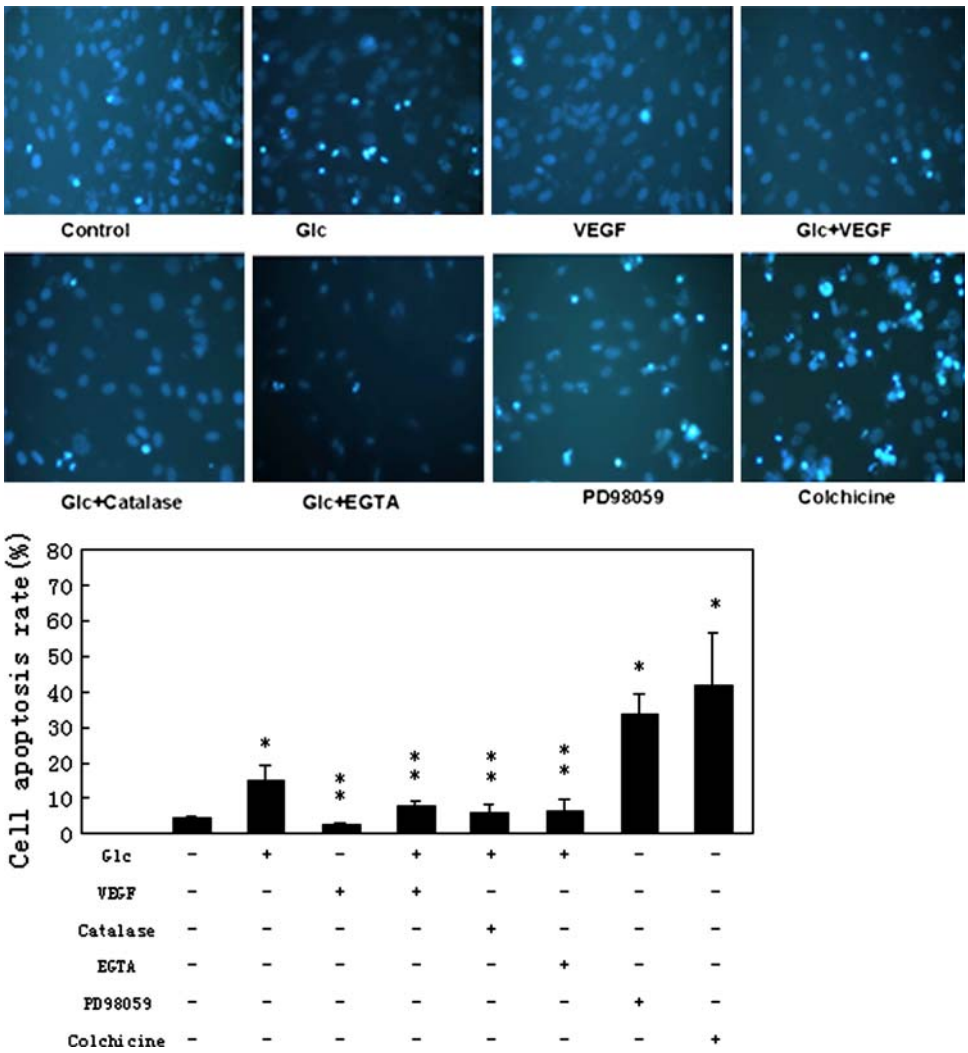

Fig. 2 Apoptosis analysis of HUVEC by Hoechst 33258 staining brightly stained nucleus of intact HUVEC is representative of cells undergoing apoptosis (mag. 400×). The reagent concentrations as following: Glc: 25 mmol/l glucose, VEGF: 20 ng/ml, catalase: 200 u/ml, EGTA: 50 nmol/l, PD98059: 40 nmol/l, and Colchicine: 25 μmol/l. * $P < 0.05$ vs. control group, ** $P < 0.05$ vs. Glc group, $n = 4$

by high glucose and decreased by VEGF (Fig. 5a). Whereas, the Bcl-2 protein amount have no obviously change (Fig. 5b). The ratio of Bax/Bcl-2 was increased two-fold by high glucose in mitochondria (Fig. 5c). Since caspase-3 plays an important role in various drug-induced apoptosis, we examined whether caspase-3 was involved in the high glucose-induced apoptosis. The cleavage band of caspase-3 was observed at 48 h after treatment with high glucose by Western blot analysis (Fig. 6a, b). Activity assay of caspase-3 showed the same changes as the cleavage (Fig. 6c). These results suggested that mitochondria pathway was involved in high glucose-induced endothelial cell apoptosis.

High glucose down-regulates VEGF in HUVEC through inhibition of p42/44 MAPK phosphorylation

RT-PCR analysis demonstrated that mRNA level of VEGF decreased when the HUVEC were treated for 72 h with high glucose, high glucose reduced VEGF mRNA level to approximate 65% of the control in 15 mmol/l group and 40% of the control in 25 mmol/l group (Fig. 7a). Similar to the changes of the mRNA level, protein level of VEGF in HUVEC decreased as glucose concentration increased (Fig. 7b) and the secretion of VEGF in medium also decreased (Fig. 7e). Compared to the control group, VEGF protein level reduced to 83% in 15 mmol/l group and 45% in 25 mmol/l group, respectively. Figure 7c showed that time point expression changes of VEGF protein after exposure to high glucose. VEGF protein level began to decrease at 6 h and lasted at least 24 h. These results suggested that high glucose exposure can down-regulate VEGF expression both at mRNA and protein level.

To further know how high glucose down-regulates VEGF, phosphorylation of p42/44 MAPK, which could regulate VEGF expression in endothelial cells, was measured. Since VEGF also regulates MAP kinase activity, we determined whether or not high glucose inhibition of MAP

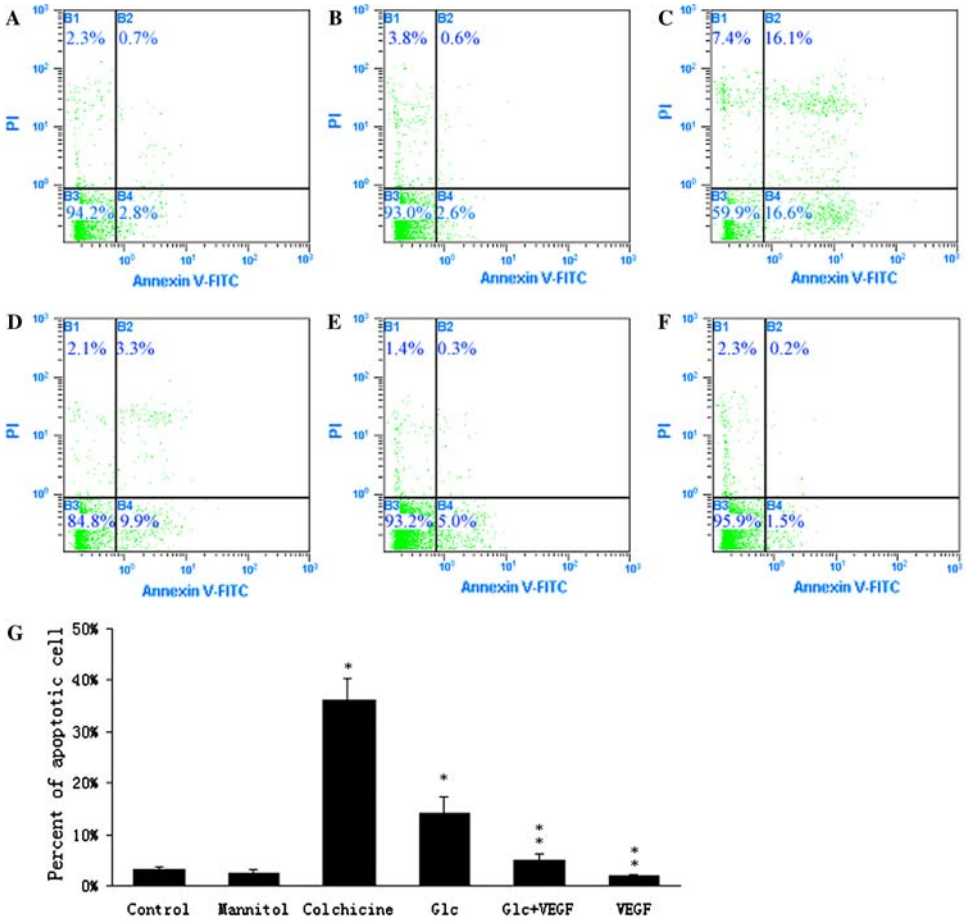

Fig. 3 Typical quadrant analysis of annexin V-FITC/PI flow cytometry of HUVEC apoptosis At least 5,000 cells were analyzed per sample. The proportion (%) of cell number is shown in each quadrant. The proportion of viable cells was shown in B3 quadrant ($FITC^-/PI^-$), early apoptotic cells shown in B4 quadrant ($FITC^+/PI^-$), late apoptotic/necrotic cells shown in B2 quadrant ($FITC^+/PI^+$). (**a**) Control group: 5 mmol/l (mM) of glucose plus 20 mM of mannitol. (**b**) Mannitol group: 25 mM of mannitol. (**c**) Colchicine group: 25 μM of colchicines as positive control. (**d**) Glc group: 25 mM of glucose. (**e**) Glc + VEGF group: 25 mM of glucose plus 20 ng/ml of VEGF. (**f**) VEGF group: 20 ng/ml of VEGF. (**g**) Percent of apoptotic cells by quantitative analysis. * $P < 0.05$ vs. control group, ** $P < 0.05$ vs. Glc group, $n = 3$

kinase activity occurred prior to the VEGF change by measuring the time course of the high glucose effect on phosphorylation of p42/44 MAPK. A 15-min treatment with high glucose resulted in a 40% inhibition of p42/44 phosphorylation compared with those in the untreated cells, the activity of p42/44 gradually returns to normal level with the time elongation (Fig. 8). As shown in Fig. 7c, the earliest VEGF decrease was detected at 6 h after high glucose exposure, while decreased p42/p44 phosphorylation appeared at 15 min after the high glucose addition (Fig. 8). In addition, inhibition of p42/44 MAPK activity using PD98059 obviously down-regulates expression of VEGF (Fig. 7d) in high glucose-treated HUVEC. These results suggested that the MAP kinase changes are upstream of the VEGF decrease.

Addition of VEGF protects HUVEC apoptosis from high glucose exposure

As shown in Fig. 3, a typical quadrant analysis of HUVEC treated with high glucose (25 mmol/l) and/or VEGF for 3 days with colchicine as positive control of apoptosis, double stained with Annexin V-FITC/PI and subjected to flow cytometry. The proportion of apoptotic cells (including early and late) for 5 mmol/l normal glucose control group was 3.3 ± 0.44% (Fig. 3a). The proportion was 14.1 ± 3.3% for 25 mmol/l glucose group (Fig. 3d). For the group of 25 mmol/l glucose plus 20 ng/ml of VEGF, the proportion was 5.0 ± 1.1% (Fig. 3e). For cells treated with normal glucose plus 20 ng/ml of VEGF, the proportion was 1.9 ± 0.3% (Fig. 3f). These results indicated that VEGF could prevent HUVEC apoptosis from high glucose exposure.

Elevated Bax/Bcl-2 ratio and caspase-3 activation in HUVEC induced by high glucose are reversed by pre-challenge with VEGF

High glucose obviously increased Bax/Bcl-2 ratio in total homogenate (Fig. 4c) and in mitochondria (Fig. 5c). Compare with the high glucose group, 25 mmol/l glucose plus 20 ng/ml VEGF significant decreased Bax expression and translocation from the cytoplasm to the mitochondrial (Figs. 4a, 5a) whereas it had no effect on Bcl-2 (Figs. 4b,

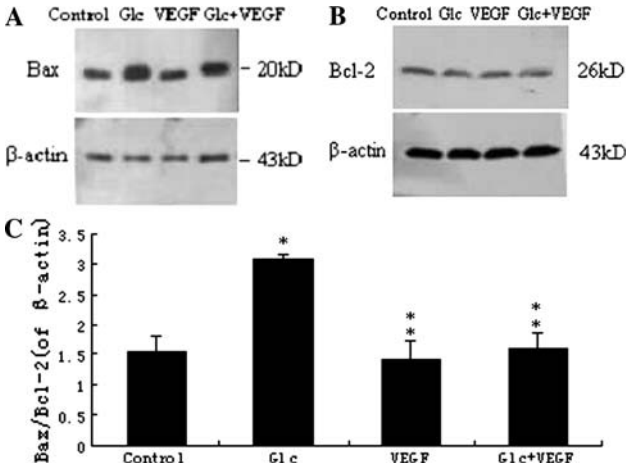

Fig. 4 Effects of high glucose and VEGF on the expression of Bax and Bcl-2 in total homogenate of HUVEC. (**a**) HUVEC were challenged with Glc or/and VEGF for 2 days, Bax levels were determined by Western blot analysis. Control: unchallenged cells, Glc: challenged with 25 mM of Glc, VEGF: challenged with VEGF, Glc + VEGF: challenged with Glc + VEGF. (**b**) Bcl-2 levels were determined by Western blot analysis. (**c**) Ratios of Bax/Bcl-2. The bands were semi-quantified with densitometry and were normalized by β-actin. The average ratios of Bax/Bcl-2 were calculated as indicated (**c**). * $P < 0.05$ vs. control group, ** $P < 0.05$ vs. Glc group, $n = 3$

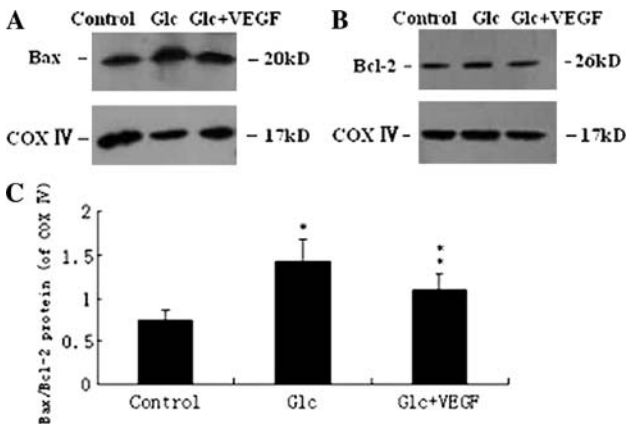

Fig. 5 Effects of high glucose and VEGF on the expression of Bax and Bcl-2 in mitochondria fraction of HUVEC. (**a**) HUVEC were challenged with Glc or/and VEGF for 2 days, Bax levels in mitochondria fraction were determined by Western blot analysis. Control: 5 mmol/l of D-glucose, Glc: challenged with 25 mM of Glc, Glc + VEGF: challenged with 25 mmol/l of D-glucose plus 20 ng/ml of VEGF. (**b**) Bcl-2 levels were determined by Western blot analysis. (**c**) Ratios of Bax/Bcl-2. The bands were semi-quantified with densitometry and were normalized by COX IV. The average ratios of Bax/Bcl-2 were calculated as indicated (**c**). * $P < 0.05$ vs. control group, ** $P < 0.05$ vs. Glc group, $n = 3$

5b). Taken together, VEGF significantly reversed the elevated Bax/Bcl-2 ratio induced by high D-glucose.

High concentration of glucose increased the cleavage of caspase-3. Addition of VEGF could significant inhibit the proportion of cleavage (Fig. 6a, b). Similarly, VEGF also

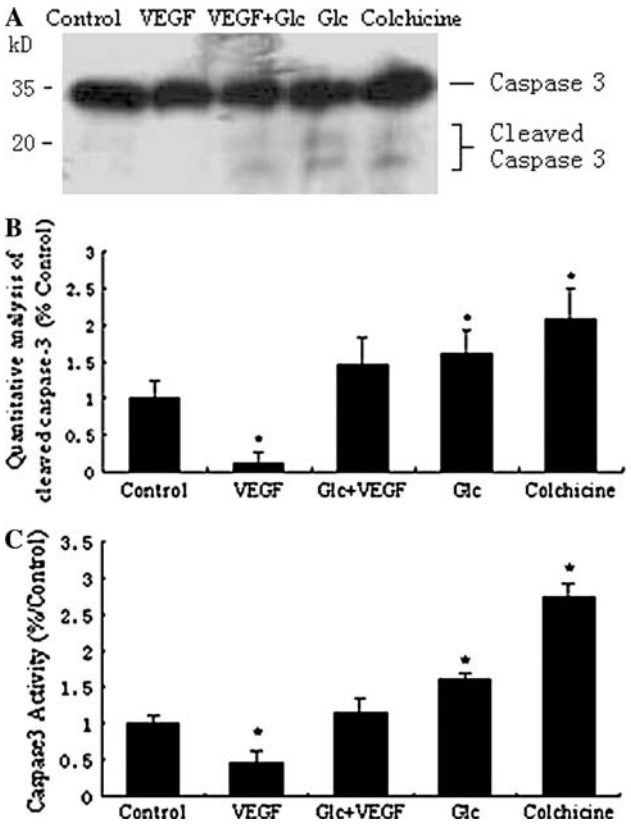

Fig. 6 Effects of high glucose and VEGF on the cleavage and activation of caspase-3 in HUVEC HUVEC were challenged with high Glc or/and VEGF for 48 h, Colchicine as positive control. (**a**) Pro-caspase-3 and its cleavage products (17 and 12 kD) were examined by Western blot analysis. (**b**) Quantitative analysis of caspase-3 cleavage products. * $P < 0.05$ vs. control group. (**c**) Caspase-3 activity was measured by a colorimetric assay based on the ability of caspase-3 to change Ac-DEVD-*p*NA into a yellow formazan product *p*NA. * $P < 0.05$ vs. control group, $n = 3$

reduced caspase-3 enzyme activity induced by high glucose in HUVEC (Fig. 6c).

VEGF attenuated ROS formation and cytoplasm calcium overload in HUVEC induced by high glucose

ROS generation is involved in high glucose-induced apoptosis. Therefore, intracellular H_2O_2 production was determined by Fenton reaction. HUVEC were exposed to high glucose (25 mmol/l) for 12–48 h. High glucose increased the formation of H_2O_2 in a time-dependent manner (Fig. 9). VEGF (20 ng/ml) treatment can significantly suppress elevation of H_2O_2 induced by high glucose (Fig. 9, $P < 0.01$).

Similarly, high glucose elicited cytosolic $[Ca^{2+}]_i$ elevation in a time-dependent manner from 24 to 96 h (Fig. 10a). VEGF (20 ng/ml) treatment almost completely suppressed $[Ca^{2+}]_i$ elevation to control level induced by high glucose (Fig. 10a, b, $P < 0.01$).

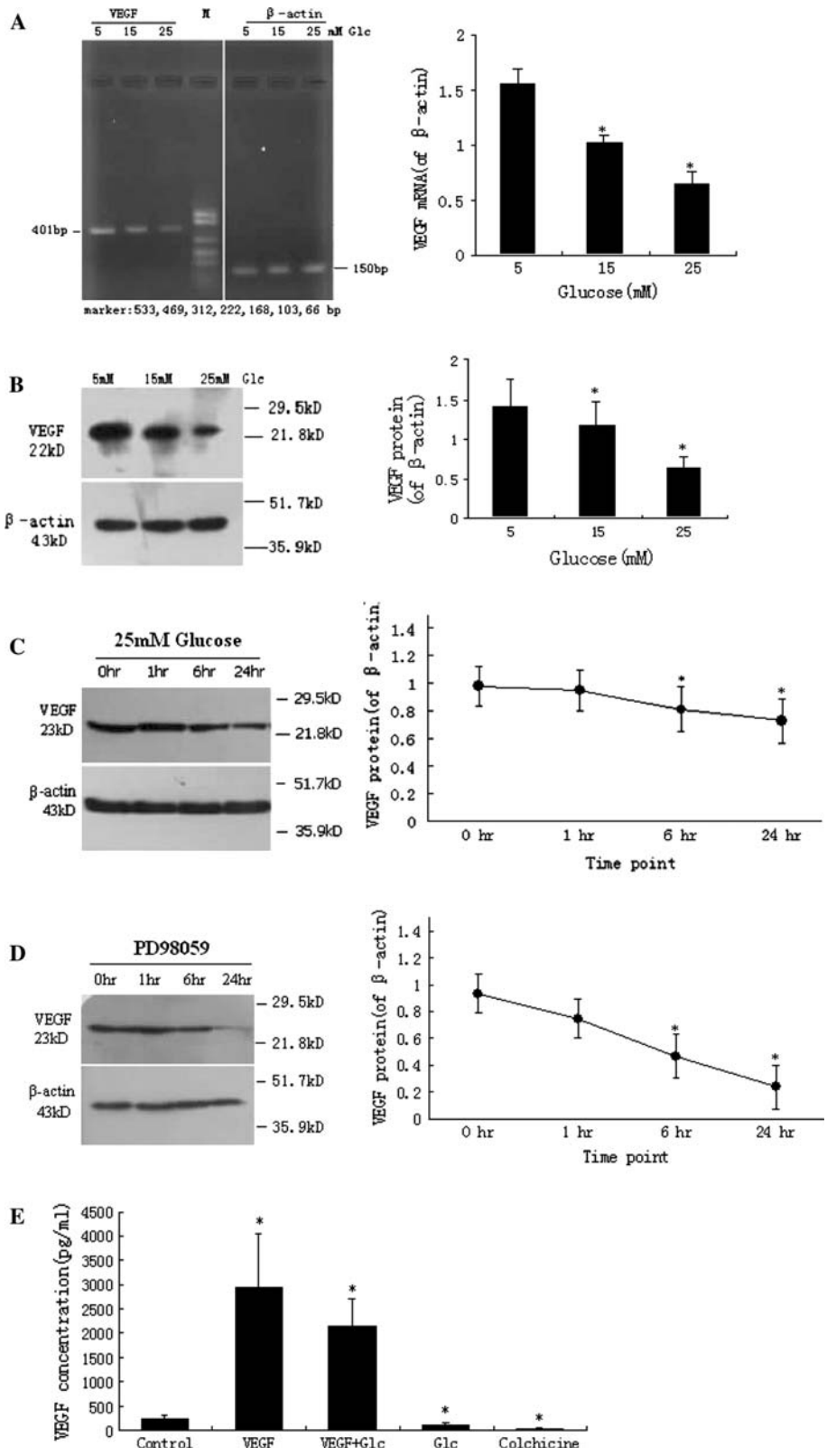

Fig. 7 Effects of high glucose and PD98059 on the expression and release of VEGF in HUVEC. (**a**) VEGF mRNA level shown by RT-PCR. Cells were cultured for 3 days in 5, 15, 25 mmol/l glucose. (left) electrophoresis gels, (right) quantitative analysis, * $P < 0.05$ vs. 5 mmol/l of glucose group. (**b**) VEGF protein level shown by Western blot analysis. (left) VEGF protein bands, (right) quantitative analysis, * $P < 0.05$ vs. 5 mmol/l of glucose group. (**c**) VEGF protein level at different time point. * $P < 0.05$ vs. 0 h group, (left) VEGF protein bands shown by Western blot, (right) quantitative analysis, * $P < 0.05$ vs. 0 h group. (**d**) Effect of PD98059, a selective inhibitor of p42/44 MAPK, on VEGF expression at different time point. * $P < 0.05$ vs. 0 h group. (**e**) Concentration of VEGF in the culture medium measured by ELISA. * $P < 0.05$ vs. control. Data averaged from three independent experiments ($n = 3$)

Same as the effect of VEGF, addition of catalase or EGTA in the medium can decrease the percent of apoptotic HUVEC (Fig. 2). These results suggested that VEGF may prevent HUVEC apoptosis from high glucose exposure at least in part through attenuation of ROS formation and cytoplasm calcium overload.

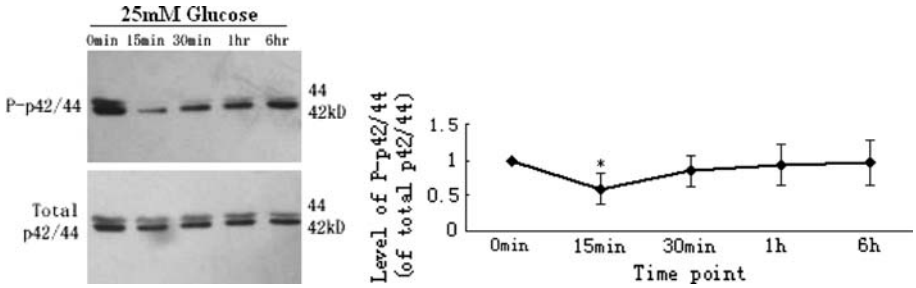

Fig. 8 Effects of high glucose on phosphorylation of p42/44 MAP kinase HUVEC were challenged with 25 mmol/l of Glc for 0 min, 15 min, 30 min, 1 h and 6 h. (left) Western blot bands, (right) quantitative analysis of phosphorylated p42/44 versus total p42/44 MAPK. * $P < 0.05$ vs. 0 min group. Data averaged from four separate cell isolates ($n = 4$)

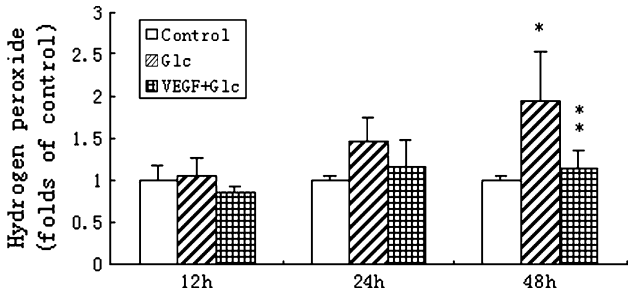

Fig. 9 Effect of VEGF on H_2O_2 formation elicited by high glucose HUVEC were exposed to high glucose (25 mmol/l) for 12, 24 and 48 h. Intracellular H_2O_2 production was determined by Fenton reaction. * $P < 0.05$ vs. control group, ** $P < 0.05$ vs. Glc group. Data averaged from three separate cell isolates ($n = 3$)

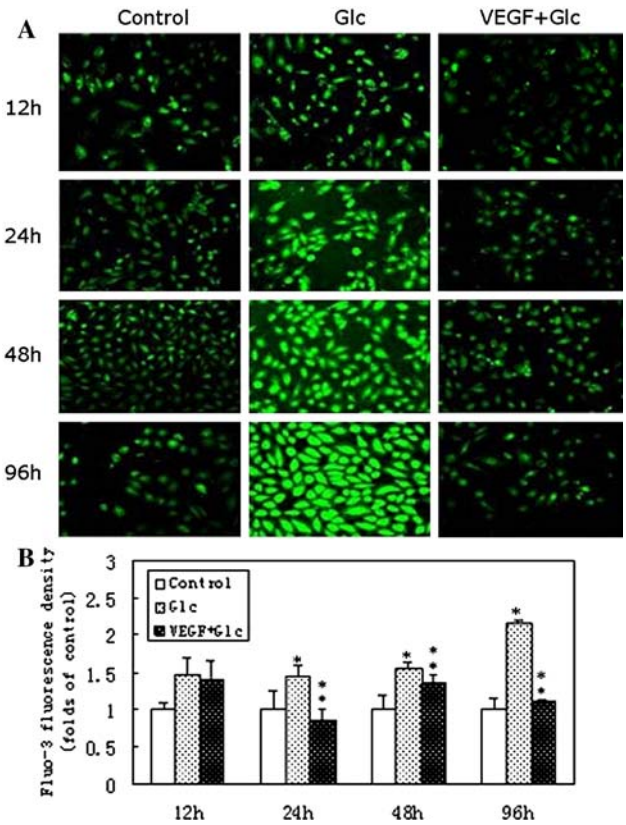

Fig. 10 Effect of VEGF on intracellular Ca^{2+} concentration elicited by high glucose. (**a**) Representative confocal microscope image of Fluo-3 staining. HUVEC were treated with high glucose (25 mmol/l) for 12, 24, 48 and 96 h. (**b**) Fluorescence density changes. * $P < 0.05$ vs. control group, ** $P < 0.05$ vs. Glc group. Data averaged from three separate cell isolates ($n = 3$)

Discussion

The present study demonstrates that elevated glucose induces apoptosis of HUVEC and down-regulates VEGF in HUVEC. Mitochondria apoptosis pathway, ROS, as well as calcium are involved in the apoptotic effect of high glucose. Addition of VEGF prevented HUVEC apoptosis from high glucose exposure by inhibition of elevated ROS generation, calcium overload, and activated of mitochondria apoptosis pathway. These results suggest that down-regulation of VEGF plays a critical role in apoptosis of endothelial cells induced by high glucose and restoration of VEGF might prevent endothelial dysfunction in the early stage of diabetes.

Constant hyperglycemia has been shown to be a direct cause of vascular endothelium damage in diabetes. Several groups [3, 4, 21] observed the cytotoxicity of high concentration of glucose to cultured vascular endothelial cells. It was shown that high concentration of glucose delayed cell replication, disturbed cell cycle and accelerated cell death in HUVEC [3]. The present study also confirmed that increased cell death in HUVEC by exposure to high glucose was caused by apoptosis. However, the molecular basis for the glucose-induced apoptosis in HUVEC is not very clear. Several pathways such as disturbed metabolic pathways, covalent modification of cellular constituents, cytotoxicity of advanced glycation products and release changes of growth factors may account for the apoptosis induced by elevated glucose. Loss of anti-proliferative substances from endothelial cells might be related to the development and progression of atherosclerosis/arteriosclerosis in diabetic patients [22, 23]. Therefore, the role of

VEGF, an endothelial cell-specific mitogen, was evaluated in apoptosis of HUVEC induced by high glucose.

There are paradoxical reports about the effect of high glucose on expression of VEGF in different type of cells or tissues. High glucose stimulates expression of VEGF in human vascular smooth muscle cells [24], diabetic retinas [25] and rat mesangial cells [26]. Conversely, others reported that high glucose decreases expression of VEGF in peripheral blood leukocytes in patients with mild to moderate type 1 diabetic retinopathy [27], in 1-week glomeruli of streptozocin-induced diabetic kidney [28], and in the murine conceptus [29]. These paradoxical results may be explained by different cell type and diabetic type, as well as different exposure time to high glucose. However, the direct effect of high glucose on the expression changes of VEGF in endothelial cells, which the key factor for endothelial dysfunction, has not been well explored. In the present study, to our knowledge, we found for the first time that high glucose down-regulates VEGF in HUVEC both at mRNA and protein level happened earlier at 6 h exposure and maintained for three days (Fig. 7). This finding is consistent with the previous reports in which decreased VEGF usually was detected at earlier course of diabetes [28], whereas, increased VEGF usually was detected in latter stage of diabetes accompanied with diabetic retinopathy or nephropathy [25, 26]. These results suggested that the down-regulation effect of high glucose exposure on VEGF expression in vascular endothelium may happen at early development stage of diabetes.

The p42/44 and p38 MAP kinase pathway have been suggested to play a role in the regulation of VEGF expression [30–32]. Our results show that high glucose inhibits the activation of p42/44 MAP kinase (Fig. 8). As VEGF itself is an activator of this pathway through its interactions with VEGF receptors [33], we have also determined if the decreased MAP kinase activation by high glucose is a cause, or a consequence, of decreased VEGF levels. In endothelial cells, high glucose displayed a fast inhibition of p42/p44 activation, and this effect occurred as early as 15 min after the exposure to high glucose, while the earliest change in VEGF levels occurred at 6 h after the exposure to high glucose, suggesting that the MAP kinase inhibition occurs prior to the down-regulation of VEGF and is unlikely a consequence of the decreased VEGF levels. Moreover, PD98059, a selective inhibitor of p42/44 MAPK, can inhibit VEGF expression (Fig. 7d) and enhance the apoptosis rate (Fig. 2) of HUVEC exposure to high glucose. These results suggest that high glucose down-regulates VEGF through inhibiting p42/44 MAP kinase activation in HUVEC. This results is similar with our previous finding that human plasminogen kringle 5, an angiogenic inhibitor, down-regulates VEGF through inhibiting p42/p44 kinase activation [34].

VEGF has a vascular protective effect by activating anti-apoptotic kinase and maintaining survival signals in endothelial cells [15–17]. Since reduced VEGF was detected in glucose-induced HUVEC, so we hypothesized that supplement of VEGF might have protective effect on apoptosis of endothelial cells induced by high glucose. In this study, cell apoptosis was analyzed by Hoechst 33258 and Annexin V/PI staining. Flow cytometry showed that the proportion of apoptotic cells for treatment with 25 mmol/l Glc plus 20 ng/ml of VEGF was $5.0 \pm 1.1\%$, whereas the proportion was $14.1 \pm 3.3\%$ for treatment with 25 mmol/l of Glc (Fig. 3). These results indicated that high concentrations of glucose resulted in a significant increase in apoptosis and VEGF can attenuate high glucose-induced apoptosis. This finding is similar to the report that hepatocyte growth factor (HGF) can attenuate high D-glucose-induced apoptosis and necrosis [35]. Therefore, the underlying mechanism for apoptosis of glucose-exposed HUVEC and anti-apoptosis of VEGF was further investigated in the present study.

The apoptosis-related proteins Bax and Bcl-2 and caspases appear to be important for the progression of apoptotic cell death in mitochondrial pathway. The homo- and heterodimerization of Bcl-2 family proteins is important for transduction and integration of apoptotic signals and control of the permeability of mitochondria and endoplasmic reticulum membranes [36]. Bcl-2 interacts with activated Bax during apoptosis in an effective manner to neutralize the proapoptotic activity of Bax, auto-activation of the apoptosis protein Bax increases mitochondrial membrane permeability and is inhibited by Bcl-2 [37]. The formation of a Bax/Bcl-2 heterodimer can inhibit Bcl-2 homodimerization [36, 37], once Bax/Bcl-2 ratio elevated can trigger apoptotic cell death. In this study, we found that high glucose significantly increased Bax protein, but not Bcl-2, and activated caspase-3 in HUVEC (Figs. 4, 6). The elevated Bax/Bcl-2 ratio could activate cleavage of pro-caspase-3 and then trigger the apoptosis of endothelial cells. These results were consistent with the previous finding in high glucose-treated human and bovine aortic endothelial cells [6, 8, 35]. Meanwhile, in the present study we demonstrated that VEGF significantly decreased Bax expression without affecting Bcl-2 level and attenuated the increase in caspase-3 activity. These findings suggest that VEGF can inhibit Bax expression and decrease Bax/Bcl-2 ratio, thereby leading to the inhibition of caspase-3 activation.

Importantly, the present studies demonstrated that VEGF significantly decreased Bax protein without affecting Bcl-2 protein. Anti-apoptotic action of VEGF through Bax deduction may be effective against not only high glucose conditions, but also other stimulation involved in the activation of the mitochondrial-mediated apoptotic

pathway [38, 39]. In this study, we found that these anti-apoptotic actions of VEGF are different with HGF. HGF attenuated high D-glucose-induced endothelial cell death through up-regulated Bcl-2 without change of Bax [35]. However, VEGF and HGF can both decrease Bax/Bcl-2 ratio and subsequently activate cleavage of caspases. In addition to classic diagrams defining the quantity, the subcellular localization of these molecules also determines the fate of cells. Analysis using fluorescence microscopy indicated that Bax protein is mainly localized within the cytosol of healthy cells [40, 41]. However, after delivery of death signals to cells in culture, Bax protein moves to the mitochondria and other membrane sites and triggers a catastrophic change of mitochondrial function [42, 43]. Nakagami et al. [35] demonstrated that after high glucose treatment, Bax protein inserts into the mitochondrial membranes, translocation of Bax protein into the mitochondrial membrane was accompanied by a significant increase in caspase-3 and 9 activity. The present study showed that the ratio of Bax/Bcl-2 was increased by high glucose and attenuated by VEGF both in cytosol and mitochondria (Figs. 4, 5). These results suggested that VEGF may inhibit the mitochondria apoptotic pathway through blocking translocation of Bax protein into the mitochondrial.

It has been reported that high glucose increased superoxide anion generation and reactive oxygen species (ROS) in HUVEC [8, 9]. The role of ROS induced by high glucose has two aspect roles: prevent apoptosis in early stage (<24 h) and induce apoptosis in late stage (~48 h). In response to high glucose stimulation, ROS/PI3 K/Akt/NO pathway plays an important role in the protection of endothelial cells from apoptosis at early stage (<24 h). However, excess and long-lasting ROS insult leads to sustained NF-κB activation, elicit JNK activation which in turn activates caspase-3 and reduction of Akt survival signaling, contributing to the apoptosis observed at late stage (~48 h) [44]. In the present experiment, intracellular H_2O_2 production was determined. The results confirmed that high glucose increases superoxide anion generation after 24 h in endothelial cells and may induce HUVEC apoptosis through ROS-dependent pathway. Conceivably, VEGF could decrease H_2O_2 production at 48 h and may thereby inhibit ROS/ NF-κB/JNK/Caspase-3 pathway.

Many evidence suggested that intracellular Ca^{2+} elevation is sufficient to induce apoptosis in many different cell types [11–14]. Cellular Ca^{2+} overload promotes mitochondrial Ca^{2+} uptake. Excessive Ca^{2+} accumulation within mitochondria is one of the primary causes for mitochondrial permeability transition, which is at least partly mediated by the opening of permeability transition pore (PTP). Mitochondrial permeability in general and the PTP complex in particular is regulated by the members of Bcl-2 family [45, 46]. Recently, Tamareille et al. [47] found that high glucose-induced apoptosis in HUVEC is associated with an increase in Ca^{2+} current resulting from Ca^{2+} entry mediated by store-operated channels, which suggest a significant role for Ca^{2+} entry in high glucose-induced apoptosis in HUVEC. Agreement with this finding, our results also demonstrated that high glucose exposure elevated intracellular $[Ca^{2+}]_i$ and the concentration increased with the time elongation accompanied with hydrogen peroxide production. Nevertheless, the effect of VEGF on intracellular $[Ca^{2+}]_i$ in endothelial cells is controversial. Some reports showed that VEGF increased $[Ca^{2+}]_i$ in endothelial cells [48, 49], but these results pertained to only short observation times (5–10 min). With elongated action of VEGF over 60 min, $[Ca^{2+}]_i$ decreased [50, 51]. Our present experiment pertains to long time action of VEGF (24–96 h), the application of 20 ng/ml VEGF almost suppressed $[Ca^{2+}]_i$ elevation to control level (Fig. 10).

Clearing off ROS or Ca^{2+} using catalase or EGTA can decrease the percent of apoptotic HUVEC induced by high glucose (Fig. 2). Inhibition of ROS or Ca^{2+} could potentialize the effect of VEGF on high glucose-induced apoptosis. These results suggested that VEGF may prevent HUVEC apoptosis from high glucose exposure at least in part through attenuation of ROS formation and cytoplasm calcium overload.

Conclusion

Taken together, the present results demonstrated that high glucose exposure can induce endothelial cell apoptosis through activation of mitochondrial apoptotic pathway, elevation of ROS and calcium overload. Down-regulation of VEGF may play a critical role in apoptosis of endothelial cells induced by high glucose. In addition to suppression of Bax/Bcl-2 ratio and caspase-3 activation, VEGF can attenuate the elevated ROS generation and calcium overload in HUVEC induced by high glucose. These finding may represent a mechanism for the anti-apoptotic effect of VEGF as summarized in Fig. 11. Although we think down-regulation of VEGF may play an important role, it is possibly partial responsible for the apoptosis of endothelial cells induced by high glucose as supplement of VEGF can only prevent part of endothelial cells from apoptosis. Combination of VEGF with other growth factors such as HGF may provide more efficient protective effect for endothelial dysfunction at early stage of diabetes development. This hypothesis still need to be further confirmed in the in vivo diabetic state in the future.

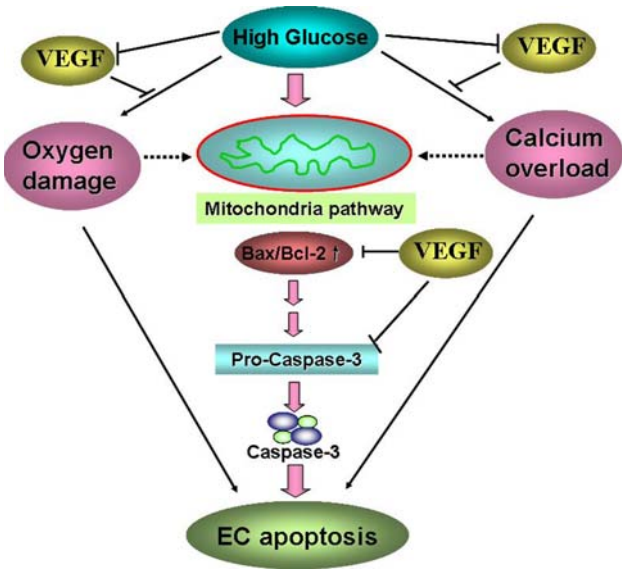

Fig. 11 Potential pathways in which high glucose induces endothelial cell apoptosis via down-regulation of VEGF. High glucose exposure can induce endothelial cell apoptosis through activation of mitochondrial apoptotic pathway, elevation of ROS and calcium overload. High glucose down-regulates VEGF in endothelial cells. In addition to suppression of Bax/Bcl-2 ratio and cleavage of caspase-3, supplement of VEGF can attenuate the elevated ROS generation and calcium overload induced by high glucose. Down-regulation of VEGF may play a critical role in apoptosis of endothelial cells induced by high glucose

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