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RESEARCH PAPER

Resveratrol ameliorates high-glucose-induced hyperpermeability mediated by caveolae via VEGF/KDR pathway

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Abstract Endothelial hyperpermeability induced by hyperglycemia is the initial step in the development of atherosclerosis, one of the most serious cardiovascular complications in diabetes. In the present study, we investigated the effects of resveratrol (RSV), a bioactive ingredient extracted from Chinese herb *rhizoma polygonum cuspidatum*, on permeability in vitro and the molecular mechanisms involved. Permeability was assessed by the efflux of fluorescein isothiocyanate (FITC)-dextran permeated through the monolayer endothelial cells (ECs). The mRNA levels, protein expressions, and secretions were measured by quantitative real-time PCR, western blot, and ELISA, respectively. Increased permeability and caveolin-1 (cav-1) expression were observed in monolayer ECs exposed to high glucose. Resveratrol treatment alleviated the hyperpermeability and the overexpression of cav-1 induced by high glucose in a dose-dependent manner. β-Cyclodextrin, a structural inhibitor of caveolae, reduced the hyperpermeability caused by high glucose. Resveratrol also down-regulated the increased expressions of vascular endothelial growth factor (VEGF) and kinase insert domain receptor (KDR, or VEGF receptor-2) induced by high glucose. Inhibition of VEGF/KDR pathway by using SU5416, a selective inhibitor of KDR, alleviated the hyperpermeability and the cav-1 overexpression induced by high glucose. The above results demonstrate that RSV ameliorates caveolae-mediated hyperpermeability induced by high glucose via VEGF/KDR pathway.

Keywords Resveratrol · Diabetes · Atherosclerosis · Hyperpermeability · Caveolae · VEGF

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Introduction

Atherosclerosis, one of the most serious cardiovascular complications of diabetes mellitus, occurs primarily in coronary arteries, lower extremities and extracranial carotid arteries. Retention or accumulation of modified macromolecules like lipoproteins and advanced glycation end products (AGE) in the subendothelial space is an initial event in the formation of atherosclerosis; however, the underlying mechanisms are not fully understood.

Hyperpermeability, the increased transport of large molecules including AGE and lipoproteins to the subendothelial space is the early response of ECs to insults such as hyperglycemia or dyslipidemia (Simionescu 2007). Caveolae, referring to the 50–100 nm sized, flask-shaped, and non-clathrin-coated invaginations of the plasma membrane, regulates the kinetics of vesicle transport. Caveolae-mediated permeability plays a major role in the

transport of large molecules across endothelium (Komarova and Malik 2010). It was reported that LDL-derived cholesterol, the major component of atherosclerotic plaque, enters the subendothelial space through the caveolae-mediated pathway (Sun et al. 2010). Results from cav-1 (caveolin-1, the key structural protein of caveolae)-deficient mice indicated that cav-1 in endothelium is essential in the translocation of LDL-derived cholesterol into the vessel wall and in the development of atherosclerosis, and this result was further confirmed in apoE-/- mice (Fernandez-Hernando et al. 2009, 2010). On the other side, vascular endothelial growth factor (VEGF), one of the strongest hyperpermeability inducers (Senger et al. 1990), plays a critical role in both physiological and pathological hyperpermeability (Bates and Harper 2002). Overexpression of VEGF was found in the progression of nephritic and ophthalmic complications in diabetes (Kim et al. 2000; Cukiernik et al. 2004). The molecular mechanism involved in the permeability alteration induced by VEGF in diabetic condition is not clear; however, there are evidences indicating that caveolae was indispensable to the process, and it was suggested that VEGF-induced permeability was mediated by caveolae (Feng et al. 1999). Report showed that VEGF increased the permeability through caveolae-mediated transcellular pathway in the blood–tumor barrier (Zhao et al. 2011). In addition, kinase insert domain receptor (KDR, or VEGF receptor-2), a receptor of VEGF that is considered mediating most of the known cellular responses (permeability included) to VEGF, locates in caveolae (Holmes et al. 2007).

Resveratrol (3,5,4′-trihydroxy-trans-stilbene, RSV), a kind of dietary polyphenol abundantly existed in red wine and Chinese herb *rhizoma polygonum cuspidatum*, might play an important role in 'French paradox' (Kopp 1998). It was reported there was an adverse link between moderate drinking of red wine and risk of heart disease (Szmitko and Verma 2005), and further the anti-atherosclerosis effect of RSV had been found (Fan et al. 2008). It was shown that RSV ameliorated the increased permeability in pulmonary artery ECs induced by side-stream cigarette smoke (Low et al. 2007). Lin reported that RSV protects against oxidized LDL-induced breakage of the blood–brain barrier (Lin et al. 2010). Limited evidence showed that RSV regulates VEGF level in vascular ECs; however, decreased VEGF level caused by RSV treatment was observed in gingival fibroblasts (Nunez et al. 2010), retinal cells (Dugas et al. 2010), tongue squamous cell carcinoma, and hepatoma cells (Zhang et al. 2005). Based on these data, we hypothesized that RSV may regulate high-glucose-induced hyperpermeability via VEGF pathway.

In all, however, the role of caveolae and VEGF in the development of atherosclerosis in diabetes and the underlying mechanisms of permeability change remain unclear. We here observed the effect of RSV on hyperpermeability induced by high glucose in monolayer aortic ECs and investigated the role of VEGF and caveolae.

Methods

Reagents and materials

Primary bovine aortic endothelial cells (BAECs) were purchased from Health Science Research Resources Bank (Osaka, Japan, no. C-003-5C). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Inc. (Grand Island, NY, USA). Twenty-four-well filtration microplate and FITC-dextran (40 kDa, anionic) were from Whatman Polyfiltronics (Whatman Inc, Clifton, NJ, USA) and Molecular Probes, Inc. (Eugene, OR, USA), respectively. Bovine VEGF ELISA Kit was from RB International Inc. (Monrovia, CA, USA); anti-KDR antibody (ab2349) was purchased from Abcam Inc. (Cambridge, UK). Anti-β–actin antibody (H-196) and anti-cav-1 antibody (sc894) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). RSV was purchased from NanJing ZheLang Inc. (NanJing, JiangSu, China); TRIZOL was from Invitrogen Inc. (Carlsbad, CA, USA), and quantitative real-time PCR kit was purchased from TAKARA Bio Inc. (Dalian, LiaoNing, China). SU5416 was purchased from Sigma–Aldrich China Inc. (Shanghai, China).

Cell culture and treatments

Primary BAECs were maintained in DMEM containing 10 % FBS at 37 °C in 5 % CO_2 as described previously (Ying et al. 2003). Six treatment groups plus one control group were included in the study; treatments were as follows: confluent cells incubated with 33 mmol/L glucose were treated with different concentrations of RSV (0, 0.1, 1, 10 μmol/L, 72 h), β-cyclodextrin (8 μmol/L, 30 min), or SU5416 (10 μmol/L, 1 h), respectively. Confluent cells incubated with 5.5 mmol/L glucose in medium were used as control group.

Cell permeability assay

Fluorescent intensity of FITC-dextran that passes through the confluent cell layer to the lower chamber was used to demonstrate the permeability. Cell permeability assay was performed as previously described (Hordijk et al. 1999). Briefly, BAECs were seeded in the upper chambers of 0.4-μm polycarbonate transwell filters of a 24-well filtration microplate. When BAECs were grown to confluence, the medium was replaced with fresh phenol red-free

DMEM. After incubation as described above, FITC-dextran (1 mg/ml) was incubated with the cells for 1 h, then the filtration microplate was removed, and the fluorescence in the medium of the feeder tray was evaluated by a multiple-mode microplate reader (Biotech, highland park, USA) at 494 nm excitation and 521 nm emission. The procedure was performed in triplicate and repeated once.

Quantitative RT-PCR

The mRNA expression levels of VEGF, KDR, and Cav-1 were analyzed with qRT-PCR. Briefly, total RNA was extracted by TRIZOL according to the manufacturer's instructions and quantified by UV spectrophotometry; samples with the value of A260/280 between 1.8 and 2.0 were used. Reverse transcription reaction (RT) was performed with 1 μg total RNA from each sample using random primers. Real-time PCR analysis was carried out using QPCR SYBR Green mix with the following parameters: 1 cycle, 95 °C, 5 s; 40 cycles, 95 °C 10 s, 57 °C, 30 s. Changes of gene expression were determined by the comparative Ct method with GADPH as reference. The primers used in the PCR were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense: TAC AAG CCC AACAAC AAG G, antisense: ACA GTG AAG GTG GTG AAG C; VEGF, sense: GGC TGC TGT AAT GAC GAA, antisense: TCC TAT GTG CTG GCT TTG; KDR, sense: ATG GGA ACC GAA ACC TA, antisense: CCT GGG CAC CTT CTA CT; Caveolin-1, sense: CTA TGG CAG AGG AAA TGA A, antisense: ACA GTG AAG GTG GTG AAG C. Six replicate samples were analyzed in each group.

Enzyme-linked immunosorbent assay

Secreted VEGF was analyzed with ELISA. An enzyme-linked immunosorbent assay was strictly according to the manufacturer's instruction. Briefly, culture supernatants were harvested, centrifuged to eliminate the impurities, and then incubated in the plate precoated with anti-bovine VEGF antibody at 37 °C for 30 min. HRP-conjugated reagent was added and the mixture was incubated at 37 °C for 30 min, and after washing, the chromogen solutions A and B were added in sequence followed by incubation at 37 °C in dark for 15 min. At last, the reaction was stopped and the absorbance was read at 450 nm within 10 min. Measurement was performed in six replicates. The result was presented as ng/L.

Electrophoresis and immunoblotting

The expression levels of KDR and cav-1 protein were analyzed using Western blot. Whole cell extracts were prepared as previously described (Ying et al. 2003). Briefly, the cells were lysed in the extraction buffer containing 50 mmol/L Tris/HCl (pH 8.0), 150 mmol/L NaCl, 1 % Nonidet-P40, 1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 0.1 mmol/L DTT, 0.05 mmol/L PMSF, 0.002 mg/ml aprotinin, 0.002 mg/ml leupeptin, and 1 mmol/L NaVO3 after stimulation. The protein concentration was quantified with BIO-RAD Dc protein assay reagent. Equal amounts of extracted proteins were separated by SDS–PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, Massachusetts, USA). Proteins were visualized using an enhanced chemiluminescence Western blotting luminal reagent (Amersham Biosciences, Little Chalfont, UK). Protein expression was visualized with a chemiluminescent detection system (Syngen, Cambridge, UK) and analyzed by Gel Pro 3.0 software (Biometra, Goettingen, Germany). Each experiment was repeated 3 times.

Statistical analysis

All quantitative data are presented as mean ± SEM. Data were analyzed by ANOVA-SNK comparisons; $P < 0.05$ was considered statistically significant.

Results

RSV down-regulated endothelial hyperpermeability induced by high glucose

Permeability of aortic endothelial monolayer cells was measured by the amount of efflux of FITC-dextran permeated through the monolayer ECs (Fig. 1). The fluorescence intensity in high glucose group was 2748 ± 88 fluorescent unit (FU), which was significantly higher than that of the control group (1990 ± 38 FU). RSV treatment reduced the fluorescence intensity in a dose-dependent manner.

SU5416 and β-cyclodextrin down-regulated endothelial hyperpermeability

To test the role of VEGF in the hyperpermeability caused by high glucose, we blocked the VEGF pathway with SU5416 (10 μmol/L, 1 h) and found that SU5416 down-regulated the permeable fluorescence significantly compared to the high glucose group (Fig. 2). Caveolae was reported to mediate macromolecule transportation. We inhibited caveolae-dependent endocytosis with β-cyclodextrin (8 mmol/L, 30 min). Compared to the high glucose group, β-cyclodextrin treatment significantly decreased the permeable fluorescence (Fig. 2).

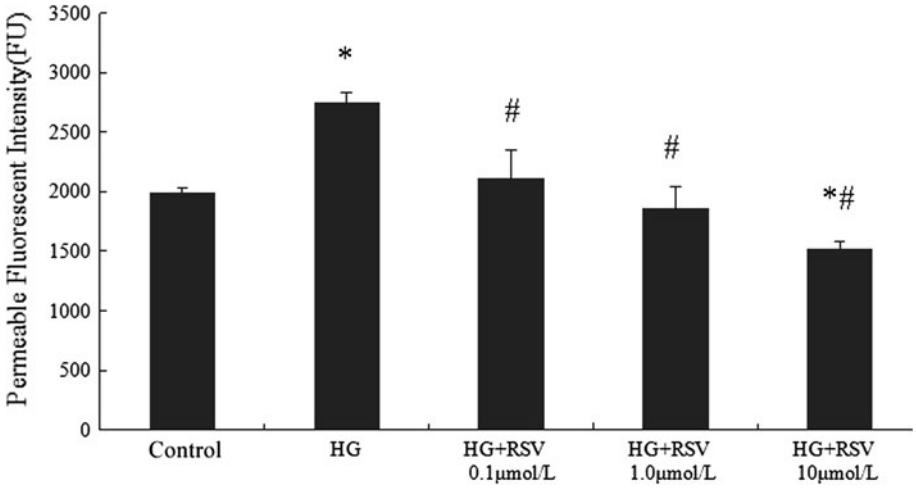

Fig. 1 Effects of RSV treatment on high-glucose-induced hyperpermeability. The result was shown by the fluorescent intensity of FITC-dextran that passes through the confluent cell layer to the lower chamber (*$P < 0.05$ vs. the control; #$P < .05$ vs. the HG group). Data are expressed as the mean ± SEM ($N = 6$)

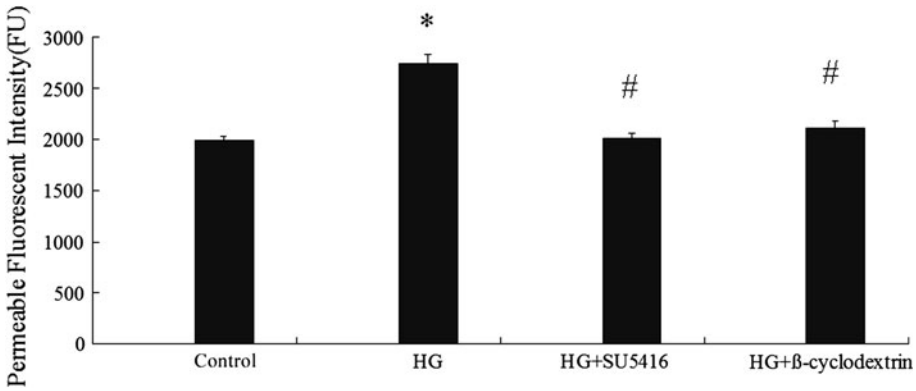

Fig. 2 Effects of β-cyclodextrin and SU5416 on high-glucose-induced hyperpermeability. Fluorescent intensity of FITC-dextran that passes through the confluent cell layer to the lower chamber was used to demonstrate the permeability (*$P < 0.05$ vs. the control; #$P < 0.05$ vs. the HG group). Data are expressed as the mean ± SEM ($N = 6$)

RSV down-regulated VEGF expression, KDR expressions, and VEGF secretion

VEGF mRNA under high glucose condition was significantly higher than that in control group, while co-incubation with RSV alleviated the increase in mRNA expression dose dependently (Fig. 3a). Besides the mRNA levels, high glucose incubation increased the VEGF secretion significantly, while the increase in secretion of VEGF was diminished in HG + RSV (1, 10 μmol/L) groups (Fig. 3b), no difference was observed in VEGF secretion level between 0.1 μmol/L RSV group and the high glucose group.

High glucose incubation up-regulated both the mRNA (Fig. 4a) and protein expressions of KDR (Fig. 4b), while treatments with three different doses of RSV lessened the increased expression of KDR.

RSV and SU5416 down-regulated cav-1 expressions

As showed in Fig. 5, compared to the overexpression of cav-1 in high glucose group, RSV incubation down-regulated the protein expression significantly. Like the effect of RSV treatment, specific blocking of VEGF-KDR pathway by SU5416 down-regulated the cav-1 expression, which suggested that caveolae could be regulated by VEGF/KDR pathway.

Discussion

The present work demonstrated that high glucose caused hyperpermeability, which can be ameliorated by RSV treatment. RSV exhibited significant effects on expressions of VEGF/KDR and cav-1, and secretion of VEGF. Evidence from inhibitors indicated that VEGF/KDR and caveolae were responsible for high-glucose-induced hyperpermeability. Furthermore, blocking of VEGF/KDR pathway down-regulated cav-1 expression and ameliorated hyperpermeability.

The prevalence of diabetes has been increasing worldwide. Over 92 million adults with diabetes and 148 million prediabetic adults make China the country with largest diabetic population (Yang et al. 2010). Atherosclerosis is the most common and serious complications in diabetes and caused 80 % of all deaths among diabetic patients (Webster and Scott 1997). Hyperglycemia is recognized as a major factor in the pathogenesis of atherosclerosis in diabetes (Laakso 1999). High glucose had been reported to

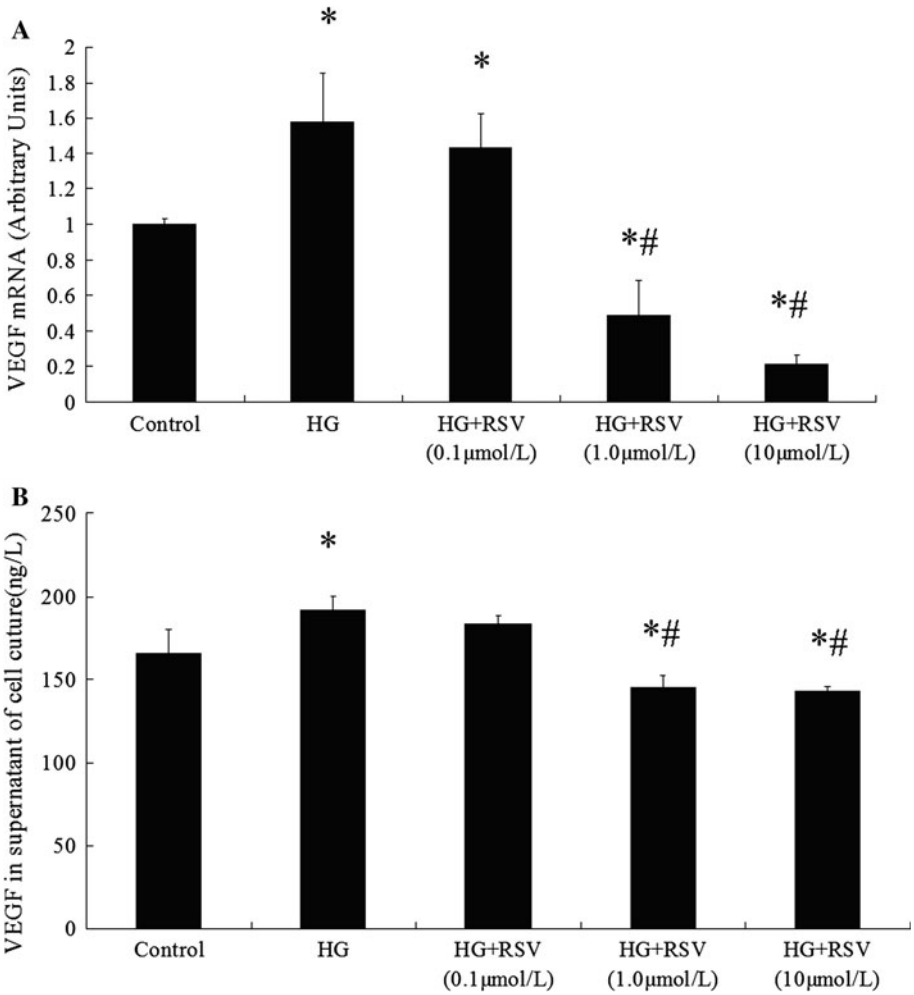

Fig. 3 Effects of RSV treatment on high-glucose-induced VEGF levels. Confluent cells were treated with different doses of RSV for 72 h, respectively. The supernatant of cell culture medium was collected for ELISA testing of secreted VEGF. (**a**) The mRNA level of VEGF after incubation. Comparative Ct method with GADPH as reference was adopted. (**b**) The secreted amount of VEGF in supernatant of cell medium, the unit is ng/L. (*$P < 0.05$ vs. the control; #$P < 0.05$ vs. the HG group). Data are expressed as the mean ± SEM ($N = 6$)

increase the permeability of ECs of umbilical vein (Dang et al. 2005) or micro-vessels (Scalia et al. 2007) in human. In the present study, high glucose incubation induced hyperpermeability in monolayer aortic ECs. Amelioration of the endothelial hyperpermeability under high glucose condition may exert a preventing effect in the initiation of diabetic atherosclerotic complications. It is well established that RSV is associated with a reduced risk of cardiovascular diseases. Although its effect on endothelial hyperpermeability was rarely reported, RSV was shown to inhibit accumulation of fatty streak, the first grossly visible lesion in the development of atherosclerosis (Auger et al. 2005). In the present study, RSV co-incubation was observed to ameliorate the high-glucose-induced hyperpermeability in aortic endothelial monolayer cells. The RSV concentrations (0.1, 1, and 10 μmol/L) used in this study were in consistence with the estimated free RSV levels of human serum, 2.06 and 0.29 μmol/L in a 70-kg man and woman, respectively (Zamora-Ros et al. 2008). The findings suggest that RSV from dietary intake may protect against high-glucose-induced hyperpermeability and be beneficial to the prevention of atherosclerotic complications of diabetes.

Caveolae, flask-shaped invaginations in the plasma membrane, were found to transport molecules from the lumen of the vessels to the subendothelial space in its original identification. Caveolae along with cav-1, the integral protein of caveolae, were recognized to be important in pathogenesis of atherosclerosis, in particular, the intimal LDL accumulation (Pavlides et al. 2012). Report showed that transportation of LDL and other macromolecules to the subendothelial space, the initial step of atherosclerosis, is mediated by caveolae (Gustafsson and Boren 2004). Furthermore, increased protein expression of cav-1 was reported in the aorta of diabetic rats (Elcioglu et al. 2010) and in the aorta of high-fat diet fed rats from our previous study (Yang et al. 2007). Cav-1 deficiency was reported to be associated with reduced atherosclerosis in apoE-/- mice (Frank et al. 2004). In the present study, increased hyperpermeability along with increased cav-1 expression was observed in high glucose incubated cells, which can be ameliorated by RSV co-treatment. And the

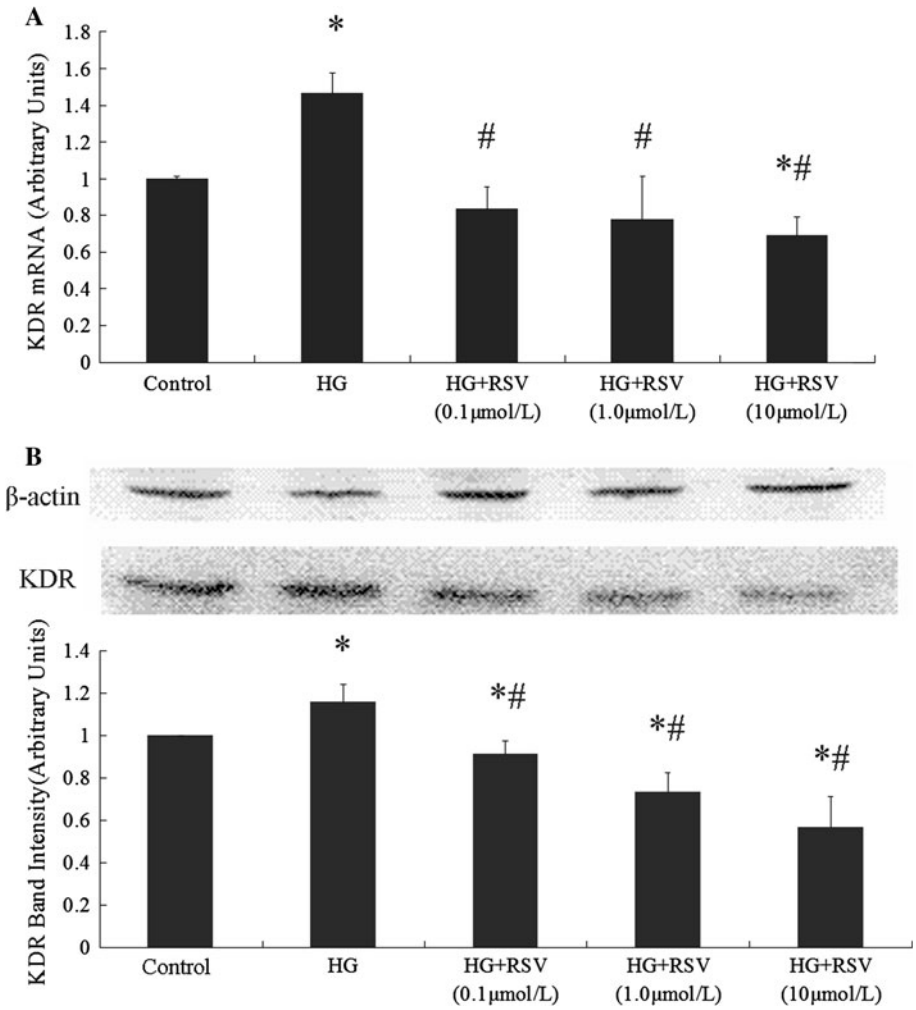

Fig. 4 Effects of RSV treatment on high-glucose-induced KDR expressions. After the treatment with different doses of RSV for 72 h, respectively, the cells were detached and lysed for total RNA and protein extraction. Comparative Ct method with GADPH as reference was adopted to analyze the mRNA level of KDR (Fig. 4a, $N = 6$). The protein expression was expressed as arbitrary units using beta–actin as reference, with the value of the control group set to 1 (Fig. 3b, $N = 3$). ($^{*}P < 0.05$ vs. the control; $^{\#}P < 0.05$ vs. the HG group). Data are expressed as the mean ± SEM

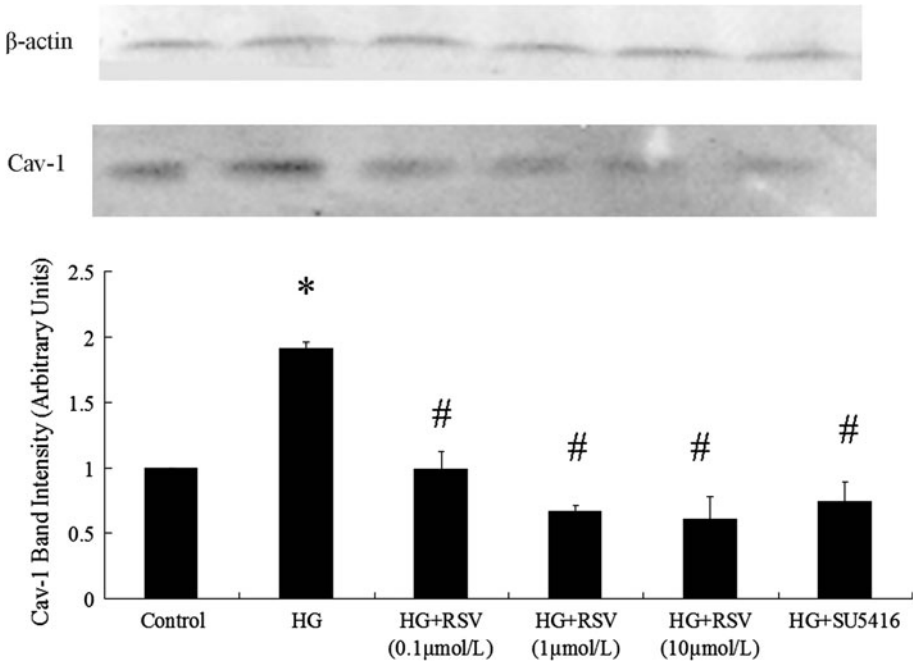

Fig. 5 Effects of RSV treatment and SU5416 on high-glucose-induced cav-1 expressions. The cells in high glucose condition were treated with different doses of RSV or SU5416, respectively. The protein expressions were detected. The result was expressed as arbitrary units using beta–actin as reference, with the value of the control group set to 1 (Fig. 5) ($^{*}P < 0.05$ vs. the control; $^{\#}P < 0.05$ vs. the HG group). Data are expressed as the mean ± SEM ($N = 3$)

same effect was observed by inhibiting caveolae-dependent endocytosis with β-cyclodextrin. Caveolae, cholesterol- and sphingolipid-rich smooth invaginations of the plasma membrane, are sensitive to cholesterol depletion. β-cyclodextrin can destroy caveolae structures (Parpal et al. 2001) by sequestering cholesterol in cultured cells (Klein et al. 1995). On the other side, Cav-1 is the key structural protein of caveolae (Drab et al. 2001). Reexpression of endothelial cav-1 in cav-/-mice demonstrated that cav-1 is indispensable to the transportation of LDL-derived cholesterol to the subendothelial space (Fernandez-Hernando et al. 2009). Further investigations using methods like morphological approaches would consolidate the conclusion.

VEGF, first identified as endothelial permeability factor, has been proved to induce hyperpermeability in vitro and in vivo. However, the mechanism of the hyperpermeability induced by VEGF is still inclusive. It was reported that VEGF increased paracellular permeability (DeMaio et al. 2004) and disrupted tight junction proteins claudin-5 (Argaw et al. 2009). Meanwhile transcellular transport by caveolae was shown to mediate the VEGF-induced endothelial permeability, and cav-1 was colocated with KDR (Feng et al. 1999; Zhao et al. 2011). VEGF induced the fission and fusion of caveolae, the increase in caveolae amount, and the increase in cav-1 expression (Chen et al. 2002). Methyl β-cyclodextrin treatment, which destroys the caveolae structure, prevented the transport of albumin, the most abundant protein in plasma (Minshall et al. 2000). As the inter-endothelial junction discontinuities are less than 3 nm and can only account for the passage of small molecules (Michel and Curry 1999), it was generally considered that macromolecules pass through the endothelium in transcellular way, most likely to be mediated by caveolae. Elevated plasma VEGF was observed in both diabetic patients with atherosclerosis and diabetic model of rabbit (Blann et al. 2002; Laakso 2008). In vitro studies demonstrated that high glucose induced VEGF in mesangial cells, glomerular podocytes, and retinal pigmented epithelial cells (Senger et al. 1990; Hoshi et al. 2002; Sone et al. 1996). Results in this study showed that high glucose incubation up-regulated VEGF and KDR expressions and increased VEGF secretions in aortic endothelial cells. Inhibition of the VEGF signaling pathway by selectively blocking of KDR down-regulated both the cav-1 expression and hyperpermeability under high glucose condition. The results indicated that VEGF/KDR pathway was responsible for high-glucose-induced hyperpermeability by regulating cav-1 expression. RSV was reported to down-regulate VEGF in diabetes (Kim et al. 2012) and inhibit the VEGF-induced pro-arteriogenic effects in a hypercholesterolemic swine model (Chu et al. 2011). Another research showed that RSV exerted anti-angiogenic effect by down-regulating KDR (Alex et al. 2010). Similar phenomenon was observed in the present study. Data show that RSV down-regulated the VEGF and KDR expressions, and VEGF secretions in cells cultured with high glucose. Furthermore, RSV also alleviated the cav-1 overexpression and hyperpermeability induced by high glucose. SU5416, selective inhibitor of KDR, resulted in same effects as RSV. Based on all above, RSV ameliorates macromolecular endothelial hyperpermeability induced by high glucose, and the amelioration is at least partially via the VEGF/KDR and caveolae pathway.

Conclusions

Our results showed that RSV alleviated the hyperpermeability induced by high glucose incubation in monolayer aortic ECs. Caveolae mediates transportation of LDL and other macromolecules to the subendothelial space, and inhibition of caveolae ameliorated the hyperpermeability induced by high glucose in the present study, which suggested that the high-glucose-induced hyperpermeability is mediated by caveolae in ECs. RSV down-regulated the VEGF, KDR, and cav-1 expressions simultaneously along with the alleviation of hyperpermeability, while selective inhibition of KDR down-regulated cav-1 expression and ameliorated the hyperpermeability. Thus, it appears that VEGF/KDR pathway is an upper stream regulator of caveolae. These result suggested that RSV ameliorated the caveolae-mediated hyperpermeability induced by high glucose via VEGF/KDR pathway. Further evidence of caveolin-mediated loss and gain function along with hyperpermeability will lead to a better understanding of the mechanisms.

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Conflicts of interest There are no conflicts of interest.

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