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ORIGINAL ARTICLE

Effects of resveratrol on NO secretion stimulated by insulin and its dependence on SIRT1 in high glucose cultured endothelial cells

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Abstract To investigate the effects of resveratrol on the secretion of NO induced by insulin in high glucose cultured primary human umbilical vein endothelial cells (HUVEC). HUVEC were treated with 1 μmol/l resveratrol for 24 h before cultured in high glucose medium for 48 h, then all cells were stimulated by 100 nmol/l insulin for 30 min. Method based on nitric acid reductase was used to analyze the NO contents in the supernatant. Cells were collected to analyze the expression of eNOS, endothelin-1, E-selectin, and SIRT1. In order to investigate the dependence of resveratrol on SIRT1, the effects of resveratrol on cells treated by SIRT1 siRNA were also examined. Compared with control cells, high glucose decreased the secretion of NO induced by insulin. Resveratrol treatment increased the expression of SIRT1 and the secretion of NO. After interfering the expression of SIRT1 using SIRT1 siRNA, the effects of resveratrol on the NO secretion induced by insulin was impaired. Resveratrol also counteracted other pro-atherosclerotic effects of high glucose, including the up-regulating roles of high glucose on the expression of endothelin-1 mRNA and E-selectin mRNA, and the down-regulating roles of high glucose on the expression of eNOS mRNA and the basal NO secretion without the stimulating of insulin. Resveratrol can improve the NO stimulating function of insulin in high glucose cultured HUVEC in SIRT1-dependent manner. Thus, our results imply that resveratrol may have the preventive roles of atherosclerosis in diabetic patients.

Keywords Resveratrol · Endothelial cells · SIRT1 · High glucose · Nitric oxide · Atherosclerosis

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Introduction

Recent changes in human lifestyle have led to a striking increase in the incidence of type 2 diabetes and its atherosclerosis [1]. A key pathogenic step in atherogenesis is the development of endothelial cell dysfunction, manifested by a reduction in bioavailability of the anti-atherosclerotic signaling molecule, i.e., nitric oxide (NO) [2]. Insulin resistance is associated with endothelial dysfunction, and a number of studies have suggested a reciprocal relationship between insulin sensitivity and endothelial cell function [3, 4]. Thus, therapies aiming at improving endothelial insulin resistance are predicted to have simultaneous beneficial effects on both metabolic and vascular function, and may decrease the occurrence of atherosclerosis in type 2 diabetes [5].

In populations with high intake of dietary cholesterol and saturated fat, moderate and regular consumption of wine, in particular red wines, is associated with a decreased incidence of cardiovascular diseases [6, 7]. This phenomenon has been described as "The French Paradox" [8]. The precise mechanisms by which red wine can prevent cardiovascular events are not completely understood but might be related to resveratrol which is rich in red wine. Resveratrol have been shown to reduce the oxidation of

low-density lipoproteins [9, 10], reduce platelet aggregation [11–13], and inhibit smooth muscular cell proliferation [14] and migration [15]. Moreover, the exposure to resveratrol is associated with increased bioactivity of NO and improved endothelial function in animal models and in humans [16–18]. However, whether resveratrol have a positive effect on the function of endothelial cells exposure to high glucose is currently unknown.

In the present study, we found that in high glucose cultured HUVEC, the secretion of NO stimulated by insulin is impaired, however, if we pretreat HUVEC with resveratrol, this consequence will be greatly improved, which is in part due to the up-regulated expression of SIRT1 stimulated by resveratrol. Moreover, we found that resveratrol can decrease the expression of endothelin-1 and E-selectin of HUVEC induced by high glucose. Thus, our results imply that resveratrol may have the preventive roles of atherosclerosis in diabetic patients.

Results

Effects of resveratrol on the morphologic appearance of endothelial cells

In our investigation, when endothelial cells were treated with resveratrol alone, a dose–response effect was observed. We found that when treated endothelial cells with 100 μmol/l resveratrol, most cells died and shed after 24 h. When changed the concentration to 10 μmol/l, 20% cells died after 24 h, and the other cells experienced great morphologic change: from the typical cobblestone-like morphologic appearance (Fig. 1a) to a thin and slender cell type (Fig. 1b). When the cells were transferred to normal culture fluid without resveratrol, cells changed back to their normal appearance. When the resveratrol concentration was 1 μmol/l, no dead cells were found and the morphologic change was also absent.

Effects of resveratrol on insulin-stimulated NO secretion in high glucose cultured endothelial cells

As shown in Fig. 2, compared with non-insulin treatment control group, insulin treatment significantly stimulated NO secretion of endothelial cells (58.907 ± 2.332 μmol/l, 83.590 ± 2.290 μmol/l, respectively, $P < 0.01$). However, in the cells cultured in 33.3 mmol/l high glucose, insulin-induced NO secretion (25.953 ± 3.027 μmol/l) was greatly impaired compared with that in insulin treatment group ($P < 0.01$). Pretreat the cells with 1 μmol/l resveratrol greatly improved the NO secretion in high glucose group (126.843 ± 9.433 μmol/l, $P < 0.01$). Moreover, the NO contents in resveratrol pretreatment group were significantly greater than that in insulin treatment group ($P < 0.01$).

Effects of resveratrol on insulin-stimulated NO secretion in SIRT1 siRNA treatment cells

As show in Fig. 3, compared with Lipo2000 group (empty Lipofectamine 2000 with no SIRT1 siRNA), the expression of SIRT1 was significantly down-regulated in SIRT1 siRNA group ($P < 0.05$). The NO secretion stimulated by insulin was also decreased followed by the SIRT1 interfere ($P < 0.05$). No difference was found between resveratrol group and Lipo2000 group ($P > 0.05$).

Effects of resveratrol on the expression of endothelin-1, E-selectin, eNOS mRNA and the basal secretion of NO in endothelial cells cultured in high glucose

As shown in Figs. 4, 5, 6, and 7, compared with control group, high glucose significantly down-regulated the expression of

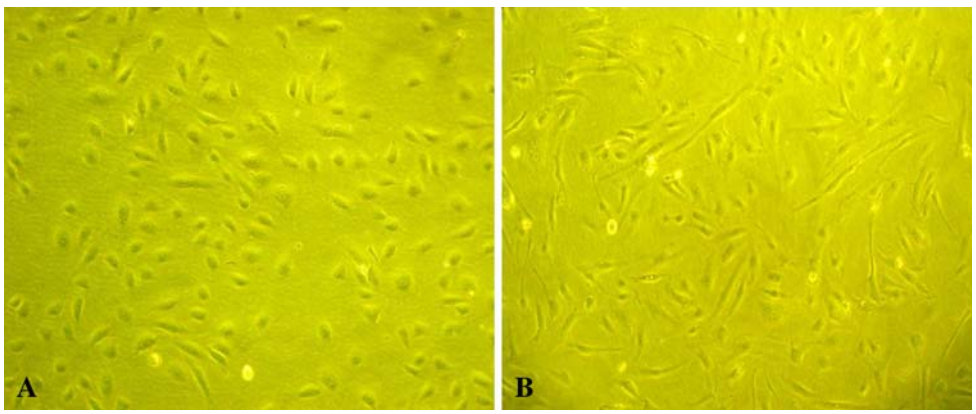

Fig. 1 Effects of resveratrol on the morphologic appearance of endothelial cells. When endothelial cells were cultured in 10 μmol/l resveratrol, 20% cells died after 24 h, and the other cells experienced great morphologic change: from the typical cobblestone-like morphologic appearance (**a**) to a thin and slender cell type (**b**). When the cells were transferred to normal culture medium without resveratrol, cells changed back to their normal appearance

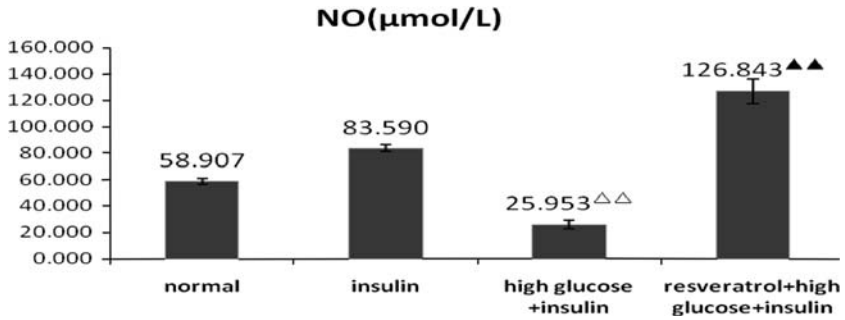

Fig. 2 Effects of resveratrol on insulin-stimulated NO secretion in endothelial cells. Values were expressed as mean ± SD ($n = 3$). $F = 199.238$, $P = 0.000$. For any comparison between these four groups: $P < 0.01$. Insulin treatment significantly stimulated the NO secretion of endothelial cells. High glucose impaired the NO stimulating role of insulin, which was counteracted by resveratrol pretreatment. $^{\Delta\Delta}$ Compared with insulin group, $P < 0.01$; $^{\blacktriangle\blacktriangle}$ compared with high glucose plus insulin group, $P < 0.01$

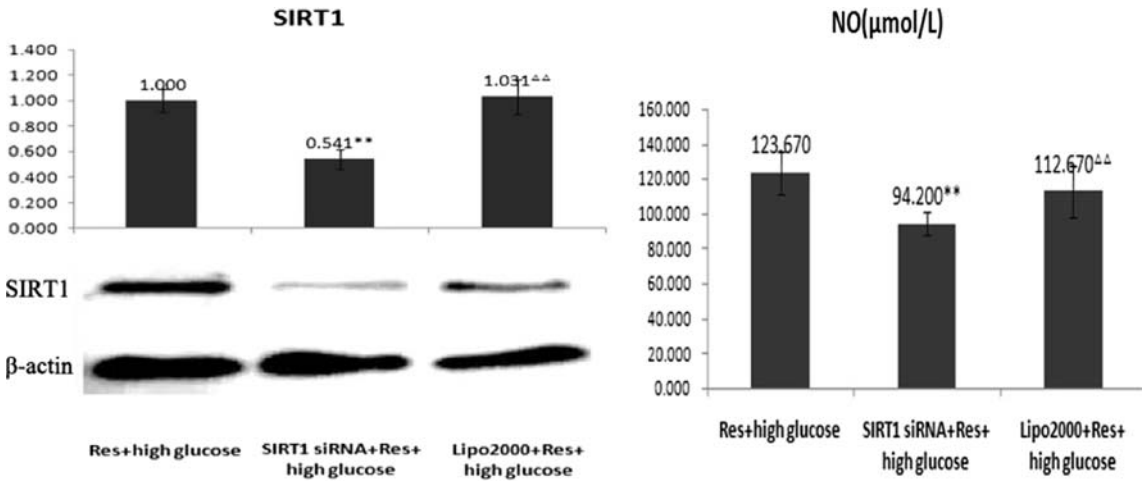

Fig. 3 Effects of resveratrol on SIRT1 expression and insulin-induced NO secretion in SIRT1 siRNA treatment cells. The data of SIRT1 is a ratio of intensities of SIRT1/β-actin. Compared with Lipo2000 group, the expression of SIRT1 was significantly down-regulated in SIRT1 siRNA group, the NO secretion stimulated by insulin was also decreased followed by the SIRT1 interfere. The interfering percentage of our SIRT1 siRNA was about 47.5%; No difference was found between resveratrol group and Lipo2000 group ($P > 0.05$). ** Compared with resveratrol plus high glucose group, $P < 0.01$; $^{\Delta\Delta}$ compared with SIRT1 siRNA group, $P < 0.01$

eNOS mRNA and the basal secretion of NO without the stimulation of insulin ($P < 0.05$), but up-regulated the levels of endothelin-1 and E-selectin mRNA ($P < 0.05$). Compared with high glucose group, resveratrol treatment significantly increased the expression level of eNOS mRNA and the secretion of NO ($P < 0.05$), and decreased the levels of endothelin-1 and E-selectin mRNA ($P < 0.05$), which partly counteracted the effects of high glucose. However, in the SIRT1 siRNA group, the level of eNOS mRNA and the secretion of NO were lower than those in resveratrol group, and the level of E-selectin mRNA was greater than that in resveratrol group, and without significant change of endothelin-1 mRNA between these two groups. Our findings indicate that the up-regulating roles of eNOS mRNA expression, the NO secretion and the down-regulating roles of E-selectin mRNA expression of resveratrol on high glucose cultured endothelial cells depend on SIRT1. But the inhibiting roles of resveratrol on endothelin-1 mRNA did not change after interfering SIRT1, which indicate that the down-regulating role of resveratrol on endothelin-1 mRNA has no relation with SIRT1. No difference was found between Lipo2000 group and resveratrol group.

Discussion

Insulin resistance is one of the most important pathophysiological mechanisms of type 2 diabetes. Over the last 20 years, great progress has been made in understanding the signal transduction pathways controlling classical metabolic actions of insulin to promote glucose uptake in skeletal muscle and adipose tissue through translocation of the insulin responsive glucose transporter GLUT4 [19]. These studies have informed more recent investigations into non-classical vascular actions of insulin that play an

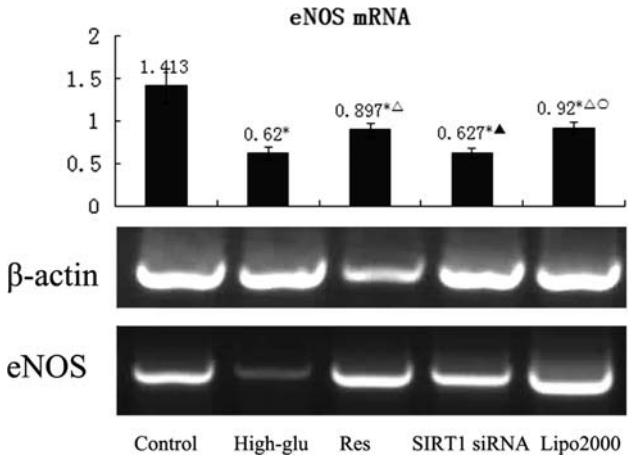

Fig. 4 eNOS mRNA level in different groups. The data is expressed as the ratio of intensities of eNOS/β-actin. Values were expressed as mean ± SD ($n = 3$). $F = 25.015$, $P = 0.000$. Compared with control group, high glucose significantly down-regulated the expression level of eNOS mRNA, which was partly counteracted by resveratrol treatment. However, in the SIRT1 siRNA group, the level of eNOS mRNA was lower than that in resveratrol group. No difference was found between Lipo2000 group and resveratrol group. * Compared with control group, $P < 0.05$; $^{\Delta}$ compared with high glucose group, $P < 0.05$; $^{\blacktriangle}$ compared with resveratrol group, $P < 0.05$; $^{\circ}$ compared with SIRT1 siRNA group, $P < 0.05$

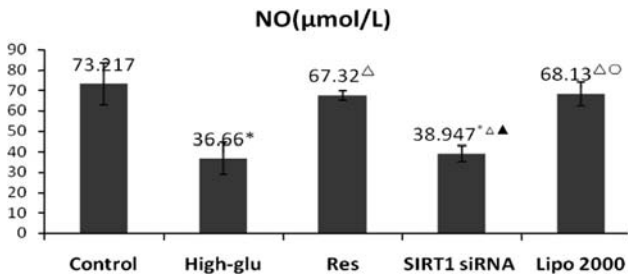

Fig. 5 Basal NO secretion in different groups. Values were expressed as mean ± SD ($n = 3$). $F = 20.756$, $P = 0.000$. Compared with control group, high glucose significantly down-regulated the NO secretion without the stimulation of insulin, which was partly counteracted by resveratrol treatment. However, in the SIRT1 siRNA group, the NO level was lower than that in resveratrol group. No difference was found between Lipo2000 group and resveratrol group. * Compared with control group, $P < 0.05$; $^{\Delta}$ compared with high glucose group, $P < 0.05$; $^{\blacktriangle}$ compared with resveratrol group, $P < 0.05$; $^{\circ}$ compared with SIRT1 siRNA group, $P < 0.05$

important role in coupling metabolic and vascular physiology [4]. In insulin-resistant conditions, impairment of shared insulin signaling pathways in metabolic and vascular tissues contributes to reciprocal relationships between insulin resistance and endothelial dysfunction. Thus, therapies aiming at improving endothelial insulin resistance are predicted to have simultaneous beneficial effects on both metabolic and vascular function, and may decrease the occurrence of atherosclerosis in type 2 diabetes [5].

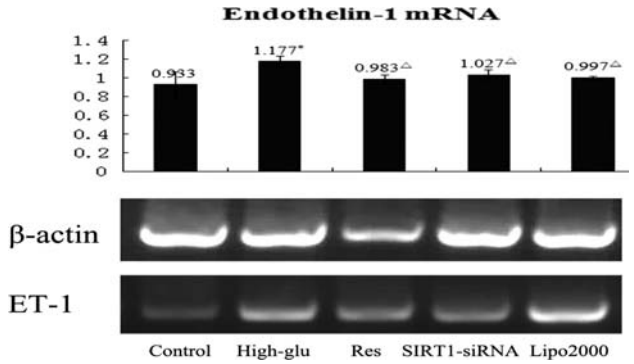

Fig. 6 Endothelin-1 mRNA level in different groups. The data is expressed as the ratio of intensities of endothelin-1/β-actin. Values were expressed as mean ± SD ($n = 3$). $F = 4.550$, $P = 0.024$. Compared with control group, high glucose significantly up-regulated the expression level of Endothelin-1 mRNA ($P < 0.05$), which was partly counteracted by resveratrol treatment. There was no difference between resveratrol group and SIRT1 siRNA group. No difference was found between Lipo2000 group and resveratrol group. * Compared with control group, $P < 0.05$; $^{\Delta}$ compared with high glucose group, $P < 0.05$

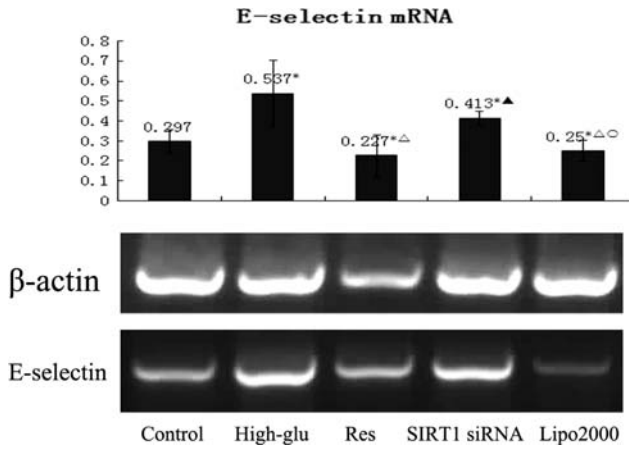

Fig. 7 E-selectin mRNA level in different groups. The data is expressed as the ratio of intensities of E-selectin/β-actin. Values were expressed as mean ± SD ($n = 3$). $F = 5.439$, $P = 0.014$. Compared with control group, high glucose significantly up-regulated the expression level of E-selectin mRNA ($P < 0.05$), which was partly counteracted by resveratrol treatment. However, in the SIRT1 siRNA group, the level of E-selectin mRNA was higher than that in resveratrol group. No difference was found between Lipo2000 group and resveratrol group. * Compared with control group, $P < 0.05$; $^{\Delta}$ compared with high glucose group, $P < 0.05$; $^{\blacktriangle}$ compared with resveratrol group, $P < 0.05$; $^{\circ}$ compared with SIRT1 siRNA group, $P < 0.05$

Resveratrol (3,5,4-trihydroxystilbene), a naturally occurring phytoalexin found in juice and red wines, has been reported to exert a variety of pharmacological effects. It has been shown to possess anti-cancer [20], anti-inflammation [21], and anti-platelet properties [22]. Resveratrol is believed to be the active ingredient in red wines that might provide answer to the "French paradox", i.e.,

the apparent compatibility of a high-fat diet with a low incidence of coronary atherosclerosis [23]. Resveratrol was also proved to possess an insulin-like effect and to promote the glucose uptake by hepatocytes, adipocytes, and skeletal muscle and hepatic glycogen synthesis in streptozotocin-induced diabetic rats [24].

In the present study, we show here that high glucose impaired the function of insulin on endothelial cells, and down-regulated the NO secretion stimulated by insulin, which proved that high glucose can induce insulin resistance in endothelial cells. But when pretreat the endothelial cells with 1 μmol/l resveratrol before culturing in high glucose, the NO stimulating role of insulin will be greatly improved. Therefore, our results imply that resveratrol has a role in improving insulin resistance of endothelial cells induced by high glucose. Our research further found that followed by the interfering of SIRT1 mRNA in SIRT1 siRNA group, the improving role of resveratrol on insulin-stimulated NO secretion was impaired, thus we conclude here that the improving role of resveratrol on insulin-induced NO secretion is dependent at least in part on its activating role of SIRT1. Our results were in accordance with the studies made by Sun et al. [25], which proved that resveratrol enhanced insulin-stimulated glucose uptake of C2C12 myotubes under insulin-resistant conditions in SIRT1-dependent manner. SIRT1 is implicated in the regulation of mitochondrial function, energy metabolism, and insulin sensitivity in rodents and more recently in humans. Rutanen et al. [26] investigated the expression of SIRT1 mRNA expression in 247 non-diabetic offspring of type 2 diabetic patients, and proved that impaired stimulation of energy expenditure by insulin and low SIRT1 expression in insulin sensitive tissues are likely to reflect impaired regulation of mitochondrial function associated with insulin resistance in humans. So together with our results, we believed that SIRT1 is important in the regulation of insulin signaling transduction in endothelial cell. Moreover, our study found that insulin-induced NO content in resveratrol plus high glucose group (126.843 ± 9.433 μmol/l) was significantly higher than that in normal control group (83.590 ± 2.290 μmol/l), and also higher than the basal NO content without the stimulation of insulin in resveratrol plus high glucose group (67.320 ± 2.341 μmol/l). Our research inferred that resveratrol may have an insulin-independent role in stimulating the NO secretion of high glucose cultured endothelial cells. As we know, oxidative stress plays an important role in the damages of endothelial cells induced by high glucose, and also is known about the anti-oxidative and free radical scavenging effects of resveratrol [10, 27]. Reactive oxygen species induced by oxidative stress can decrease the level of bioavailable NO by binding with NO. In a paper published recently, Xu et al. [28] reported that resveratrol has a role in suppressing high glucose-induced generation of superoxide anion in endothelial cells; thus, resveratrol may indirectly increase the NO content through its anti-oxidative role.

In endothelial cells, endothelial nitric oxide synthase (eNOS) catalyzes the conversion of the substrate L-arginine to the products NO and L-citrulline. Our research shows that resveratrol also has a positive role in up-regulating the expression level of eNOS mRNA and the basal secretion of NO without the stimulation of insulin, and the role was SIRT1-dependent. In accordance with our results, Xu et al. [28] proved that in HUVEC, resveratrol (10–100 μM) enhanced phosphorylation of eNOS at Ser1177 and NO production in a concentration-dependent manner. Thus, resveratrol may increase NO secretion by simultaneously increasing eNOS expression and its activity. Moreover, resveratrol was also proved to have a role in improving the expression of endothelin-1, E-selectin mRNA in endothelial cells cultured by high glucose. Endothelin-1 is a vasoconstrictor secreted by endothelial cells that opposes vasodilator actions of NO [29]. Our research shows here that high glucose significantly up-regulated the level of endothelin-1 mRNA, which can be counteracted by resveratrol pretreatment in a SIRT1-independent manner. Endothelial expression of cellular adhesion molecules such as E-selectin is critical in modulating cell–cell interactions between circulating inflammatory cells and vascular endothelium. Our research shows that resveratrol can counteract the stimulating role of high glucose on the expression of E-selectin mRNA, which is SIRT1-dependent. Hence, we concluded here that resveratrol has a protective role on the function of endothelial cells cultured in high glucose.

Recently, resveratrol have been proved to have toxic effects on many tumor cells. Although the cytotoxic roles of resveratrol were proved to be tumor cell selectively, resveratrol was also found have some toxic effects on other cells such as endothelial cell, lymph cells, and cartilage cells. So the safety of resveratrol has obtained great concern. However, resveratrol may act differently at different concentration, and was found to have protective roles on normal cells at a lower concentration. In our investigation, we found that when treated endothelial cells with 100 μmol/l resveratrol, most cells died and shed after 24 h. When changing the concentration to 10 μmol/l, 20% cells died after 24 h, and the other cells experienced great morphologic change: from the typical cobblestone-like morphologic appearance to a thin and slender cell type. When the cells were transferred to normal culture fluid without resveratrol, cells changed to their normal appearance. We have no idea about why and how resveratrol change the morphologic appearance of endothelial cells, but in our investigation, we found that the same morphologic appearance also happened to the cells lacking

nutrition. So the morphologic change of endothelial cells may reflect a kind of damage induced by resveratrol. When the resveratrol concentration was 1 μmol/l, no dead cells were found and the morphologic alteration was also absent. Consistent with these findings, Sun et al. [25] found in their study that when the resveratrol concentration is higher than 10 μmol/l over a 24-h duration, it produces toxic effects in differentiated C2C12 cells. A recent investigation has also suggested that high concentrations of resveratrol (100 μmol/l) over a 24-h duration can induce apoptosis to human embryonic kidney 293 cell [30]. Different concentration of resveratrol may function in different manner. However, low effective concentrations of resveratrol are of great therapeutic importance since lower concentrations mean greater biological safety and lower pharmaceutical cost.

In conclusion, resveratrol can improve the NO stimulating function of insulin in high glucose cultured HUVEC in SIRT1-dependent manner, which inferred that resveratrol may have anti-atherosclerosis role in diabetic patients.

Materials and methods

HUVEC culture

Cell culture reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO) except when otherwise specified. HUVEC were isolated from human umbilical cord vein with 0.25% pancrease enzyme, and then were cultured in DMEM supplemented with 20% FBS. HUVEC were passaged when they reached 80% confluence and passages between 2 and 5 were used for all experiments.

Resveratrol treatment

A stock concentration of 100 mM resveratrol in 50% DMSO was made up fresh each time and diluted in culture medium to the desired concentration. The concentration of DMSO was kept constant at no more than 0.1% throughout the experiments. Controls received the same amount of DMSO.

Cell treatments

Cells were treated with 1 μmol/l resveratrol for 24 h before culturing in 33.3 mmol/l high glucose for 48 h, and then the cells were stimulated by 100 nmol/l insulin for 30 min. In order to investigate the dependence of resveratrol on SIRT1, cells were treated with SIRT1 siRNA before resveratrol/high glucose treatment. The expression of eNOS, E-selectin, endothelin-1 mRNA were analyzed using RT-PCR. The expression level of SIRT1 protein was analyzed using Western blot. Supernatant of cells were collected for the analysis of NO contents using Nitric oxide Assay kit based on nitric acid reductase method.

Western blot analysis

The control and treated cells were lysed in lysis buffer (10 mM Tris–HCl, pH 8, 120 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 10 g/ml pepstatin, and 20 g/ml leupeptin) at 4°C for 20 min, and the lysate was then centrifuged for 10 min in a microcentrifuge. Aliquots of lysates were fractionated on a 12% SDS–PAGE. After electrotransfer, the nitrocellulose membranes were incubated with antibodies to SIRT1 (Sigma, USA) followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham, Buckinghamshire, UK). The immunoreactive protein bands were visualized by enhanced chemiluminescence (ECL; Amersham International). Each blot shown in the figures is representative of at least three experiments. Protein quantification was performed by Quantity One software (Bio-Rad), and the intensity values were normalized to β-actin.

Semi-quantitative reverse transcription PCR

RNA extraction from cells was performed with TRIzol reagent (Invitrogen). The integrity of the RNAs was confirmed on 1% denatured agarose gel electrophoresis. A total of 5 μg RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, USA) in the presence of primers. For normalization of RNA loading, the house-keeping gene *β-actin* was also amplified from each sample. PCR amplification was performed using the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s (for eNOS, it was 59°C for 30 s), 72°C for 30 s, and final extension at 72°C for 8 min. The PCR primers were: E-selectin: upstream: 5′ CTATTTGTTTTCTTCTG TATGTTAG 3′, downstream: 5′ CTCTGCTGTTCTGATC CTTATC 3′; Endothelin-1: upstream: 5′ CTAAATCCTAG CCTCGTAGAAG 3′, downstream: 5′ CTAAAGTCATTA CCTTGACAGGC 3′; eNOS: upstream: 5′ GTCTGCGG CGATGTTACCATG 3′, downstream: 5′ CAACAGGCCT AGAGAGGGAAAAGA 3′; β-actin: upstream: 5′ CGTG ACATTAAGGAGAAGCTG 3′, downstream: 5′ CTAGAA GCATTTGCGGTGGAC 3′. After amplification, 25 μl of PCR product was run on a 2% agarose gel and visualized by ethidium bromide staining.

siRNA process

The target sequence of human SIRT1 for RNAi was described elsewhere [31]. Endothelial cells were seeded at

5×10^5/60 mm dish and were transfected with 400 pmol of RNA double-stranded oligonucleotides using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. Following transfection, cells were allowed to recover for 24 h before being exposed to resveratrol/high glucose.

NO measurement

The NO level was assessed by NO Detection Kit based on the method of nitrate reductase (Nanjing Jiancheng Bioengineering Institute, China), according to the manufacture's specification.

Statistical analysis

SPSS11.5 statistical software was used for data processing. Comparison of numerical variable data was conducted with the single factor analysis of variance (ANOVA); differences between individual group were analyzed by SNK-q test. Differences were considered statistically significant at $P < 0.05$.

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