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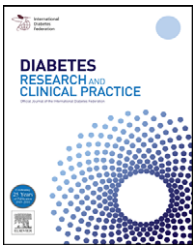

# L-arginine attenuates high glucose-accelerated senescence in human umbilical vein endothelial cells

Weili Zhong [a], Guoliang Zou [b], Jianqiu Gu [a], Jin Zhang [a,*]

[a] Department of Endocrinology, First Affiliated Hospital of China Medical University, Shen Yang, China
[b] Department of Cardiology, First Affiliated Hospital of China Medical University, Shen Yang, China

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ABSTRACT

*Objective:* Endothelial dysfunction is a key event in the onset and progression of atherosclerosis associated with diabetes. Increasing cell senescence may lead to endothelial dysfunction and contribute to vascular complications. Therefore, we aimed to elucidate the possible role and mechanism of L-arginine in preventing cell senescence induced by high glucose.
*Methods:* HUVECs were respectively cultured under normal control glucose (5.5 mM), high glucose (33 mM), co-incubation with L-arginine (800 μM)and high glucose, and senescence was identified by β-galactosidase staining, change of cell cycle and telomerase activity. Akt and eNOS activity was analyzed by western blot.
*Results:* High glucose significantly increased number of β-galactosidase-positive stained cells, inhibited telomerase activity, increased proportion of cells in the $G_0/G_1$ phase and reduced proportion in the S phase, and decreased NO synthesis. L-arginine significantly attenuated these senescent alterations. Furthermore, high glucose induced a decrease in Akt and eNOS activity, and L-arginine prevented the decrease in activity. The PI3K inhibitor LY294002 or eNOS inhibitor L-NAME attenuated anti-senescence effect of L-arginine.
*Conclusion:* L-arginine may have an anti-senescence effect via the PI3K/Akt pathway in HUVECs exposed to high glucose and it might be a therapeutic agent for diabetic vascular complications.



## 1. Introduction

Several conditions, including dyslipidemia, hyperglycemia, and increased oxidative stress, have been proposed to explain the acceleration of atherosclerosis in diabetes, [1]. Endothelial dysfunction might be the beginning of atherosclerosis in diabetes mellitus [2–4]. Because the onset and progression of vascular complications are delayed in diabetic patients with good glycemic control, hyperglycemia is thought to be a key factor in the development of endothelial dysfunction [3]. Some findings show that cell senescence is an important factor contributing to the increased vascular risk associated with aging [5], and high glucose can accelerate cell senescence [6]. Therefore, prevention of high glucose-induced endothelial-cell senescence may be a new target to treat diabetes-associated atherosclerosis.

Some cell senescent characteristics are increased senescence-associated β- galactosidase (SA β-gal) activity [7], inhibited telomerase activity [8], and depressed cell proliferation [9] in the senescence stage. The most evident hallmarks are increased oxidative stress and reduced nitric oxide (NO) bioavailability [10,11]. The latter may result from decreased expression and activity of NO synthase [12,13]. By activating the downstream serine/threonine kinase Akt, PI3K can

* *Corresponding author*. Department of Endocrinology, First Affiliated Hospital of China Medical University, NO.155 North Nanjing Street, Heping District, Shen Yang 110001, China. Tel.: +86 13504186633; fax: +24 83283073.
E-mail address: zhangjininsy@gmail.com (J. Zhang).
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promote cell survival and activate eNOS, which leads to NO production [14]. The PI3K/Akt/eNOS/NO pathway plays an important role in preventing high glucose-induced endothelial cell injury [15], and L-arginine prevents the onset of endothelial aging under high glucose [16].

We aimed to confirm whether L-arginine delays high glucose-induced cell senescence via the PI3K/Akt/eNOS/NO pathway in human umbilical vein endothelial cells (HUVECs).

## 2. Materials and methods

### 2.1. Materials

L-arginine, L-NAME and LY294002 were from the Beyotime Institute of Biotechnology (Shanghai). D-glucose and propidium iodide (PI) were from Sigma (Steinheim, Germany). Antibodies against phospho-Akt (Ser-473) (p-Akt) and phospho-eNOS (Ser-1177) (p-eNOS) were from Cell Signaling Technology (Beverly, CA, USA). Antibodies against Akt and eNOS were from Beyotime Institute of Biotechnology.

### 2.2. Cell culture

HUVECs (C-003-5C, Cascade Biologics) were cultured in normal glucose (5.5 mM) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells at passages 3∼4 were used.

*Study protocol*. After serum-starvation for 24 h, HUVECs were incubated in normal glucose (control group, 5.5 mM), high glucose (HG group, 33 mM), L-arginine (800 μM, chosen by preliminary experiment) plus high glucose, pretreated with LY294002 (20 μM) for 2 h before L-arginine and high glucose or L-arginine and normal glucose, pretreated with L-NAME (100 μM) for 2 h before L-arginine and high glucose or L-arginine and normal glucose for 24 h.

### 2.3. Detection of senescence

HUVECs were fixed and stained for SA β-gal activity with use of the Senescence β-Galactosidase Staining Kit (Beyotime Institute of Biotechnology). The percentage of SA β-gal positive cells was determined by counting, the number of blue cells within a sample of 1,000 cells.

### 2.4. Telomerase activity

Quantitative determination of telomerase activity involed use of the TeloTAGGG telomerase PCR ELISA$^{PLUS}$ Kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. An amount of 2 μg protein was used for PCR with the telomeric repeat amplification protocol assay to measure telomerase reverse transcriptase (TERT).

### 2.5. Cell cycle

Detection of the cell cycle involved flow cytometry (PI staining method), and changes in cell cycle were analyzed by the proportion of cells in the G0/G1, S, and G2 / M phases. An amount of $1*10^6$ cells was collected, then centrifuged for 5 min at 300 × g and fixed overnight with 70% alcohol at 4 °C. Cells were centrifuged to remove alcohol, and washed twice with cold phosphate-buffered saline (PBS), then PI stain was added to a final concentration of 50 μg/ml and protected from light for 30 min at 4 °C. Cell flow speed was not more than 60 cells/min and we selected at least 10, 000 cells. Data were analyzed by Moldifit 2.0 (BD company).

### 2.6. Western blotting

Cells were lysed in RIPA lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. Proteins were separated by 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes and washed 3 times with 1*TBS (pH 7.6) buffer. The membranes were soaked in 5% nonfat dry milk for 2 h and incubated overnight at 4 °C with the anti-p-Akt (Ser-473) or anti-p-eNOS (Ser-1177) polyclonal antibody (diluted 1:1000). After incubation with horseradish peroxidase-conjugated secondary antibody (diluted 1:5000) for 2 h at room temperature, the immune complexes were visualized by enhanced chemiluminescence methods, the band intensity was measured and quantitated. The p-Akt or p-eNOS band intensity was relative to the β-actin band intensity. The resulting images were analyzed by Scion Image software.

### 2.7. Measurement of NO production

Total NO production in culture medium was determined by measuring the concentration of nitrate and nitrite, a stable metabolite of NO, by modified Griess reaction method. The procedure involed use of the Total Nitric Oxide Assay Kit (Beyotime Institute of Biotechnology).

### 2.8. Statistical analysis

Data obtained from four separate experiments are presented as mean ± S.E. Statistical significance was analyzed by repeated-measures ANOVA with an LSD post hoc test or ANOVA for multiple comparisons. Data for 2 groups were analyzed by *t* test. SPSS v 16.0 (SPSS Inc., Chicago, IL,USA) was used for analysis. A $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. L-arginine attenuated SA β-gal activity under high glucose condition

High glucose (33 mM) significantly enhanced SA β-gal activity in HUVECs, by 251.9%, compared with the control group and L-arginine attenuated SA β-gal activity, by 62.1% compared with the HG group (Fig. 1). The effect of L-arginine attenuating the high glucose-induced SA β-gal activity was inhibited by treatment with the PI3K/Akt inhibitor LY204002 or eNOS inhibitor L-NAME, which increased SA β-gal activity by 132.3% or 151.0% as compared with the L-Arg+HG group (Fig. 1).

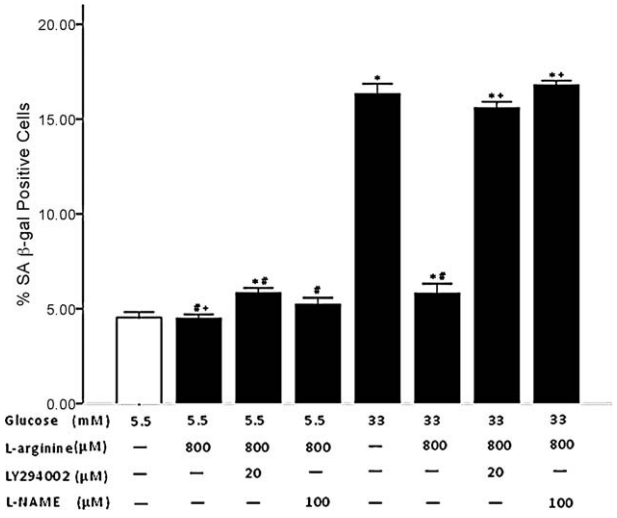


**Fig. 1 – L-arginine decreased SA β-gal activity induced by high glucose in HUVECs. Endothelial senescence was detected by SA β-gal-positive cells. HUVECs were incubated with different mediators for 24 h. Values are mean ± S.E.M form 4 independent experiments. $^{*}p < 0.05$ compared with normal glucose group. $^{\#}p < 0.05$ compared with high glucose group. $^{+}p < 0.05$ compared with L-Arg plus high glucose group.**

### 3.2. *L-arginine enhanced telomerase activity under high glucose condition*

Inhibited telomerase activity is one of the characteristics of senescent cells. In our research, high glucose significantly attenuated telomerase activity in HUVECs, by 45.4% as compared with the control group over 24 h (Fig. 2). However, L-arginine enhanced telomerase activity in HUVECs, by 194.5% as compared with the control group (Fig. 2). The effect of L-arginine enhanced high glucose-induced attenuation of telomerase activity was inhibited by LY204002 or L-NAME (Fig. 2), being inhibited by 77.2% or 82.9%, respectively.

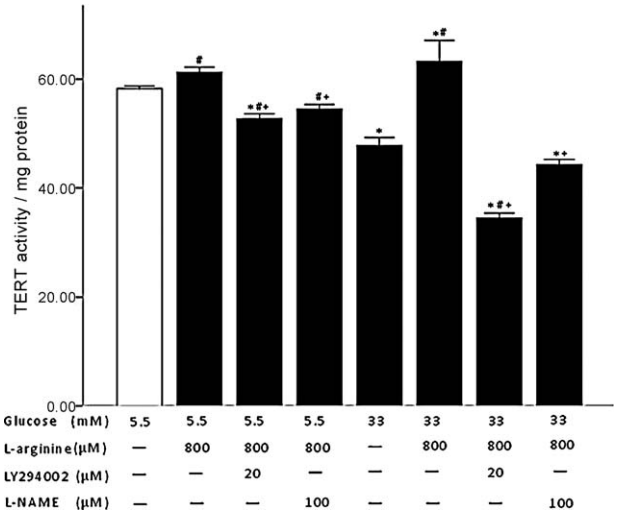


**Fig. 2 – L-arginine enhanced telomerase activity induced by exposure to high glucose in HUVECs. Telomerase activity was measured by telomerase reverse transcriptase (TERT) activity. HUVECs were incubated with different mediators for 24 h. Values are mean ± S.E.M for m 4 independent experiments. $^{*}p < 0.05$ compared with normal glucose group. $^{\#}p < 0.05$ compared with high glucose group. $^{+}p < 0.05$ compared with L-Arg plus high glucose group.**

### 3.3. *L-arginine regulated the cell cycle under high glucose condition*

High glucose increased the proportion of HUVECs in the $G_0/G_1$ phase and decreased that in the S phase, thereby inhibiting cell proliferation (Fig. 3). L-arginine regulated the cell cycle by increasing the proportion of cells in the S phase cells and accordingly decreasing that in the $G_0/G_1$ phase, thus promoting cell proliferation. This effect was inhibited by LY204002 or L-NAME (Fig. 3).

### 3.4. *L-arginine promoted Akt and NOS activity under high glucose condition*

To explore the possible mechanism of the anti-senescent effect of L-arginine on high glucose-induced HUVECs, we investigated the effect of L-arginine on Akt activity. High glucose decreased the ratio of p-Akt (Ser-473) to Akt by 38.9% as compared with the control group, L-arginine promoted the ratio of p-Akt to Akt by 47.1% as compared with the HG group (Fig. 4 A). The protective effect of L-arginine was inhibited by LY204002 or L-NAME, by 30.0% or 36.3%, respectively.

The PI3K/Akt signaling pathway is known to regulate eNOS activity, and activation of Akt has been shown to stimulate phosphorylation of eNOS at Ser-1177 [17]. Therefore, we investigated the effect of L-arginine on the ratio of p-eNOS (Ser-1177) to eNOS in HUVECs. High glucose decreased this ratio by 49.0% as compared with the control group, so eNOS activity was decreased. L-arginine prevented this negative effect by increasing the ratio of p-eNOS to eNOS, by 78.8% as compared with the HG group (Fig. 4 B). However the protective effect of L-arginine was inhibited by LY204002 or L-NAME (Fig. 4 B), by 40.0% pr 43.2%, respectively.

### 3.5. *L-arginine increased NO production under high glucose condition*

To provide insight into the cellular mechanism of L-arginine-induced decrease in high glucose-accelerated endothelial cell senescence, we measured the production of NO in conditioned media. High glucose decreased the NO production by 67.9%, as compared with the control group (Fig. 5). However, L-arginine significantly increased NO synthesis, by 715.5%, as compared with the HG group. The L-arginine-induced increase in NO production was reversed in the presence of LY294002 or L-NAME, by 79.7% or 82.0%, respectively (Fig. 5).

## 4. Discussion

Endothelial dysfunction is a key event in the onset and progression of atherosclerosis associated with diabetes [2],

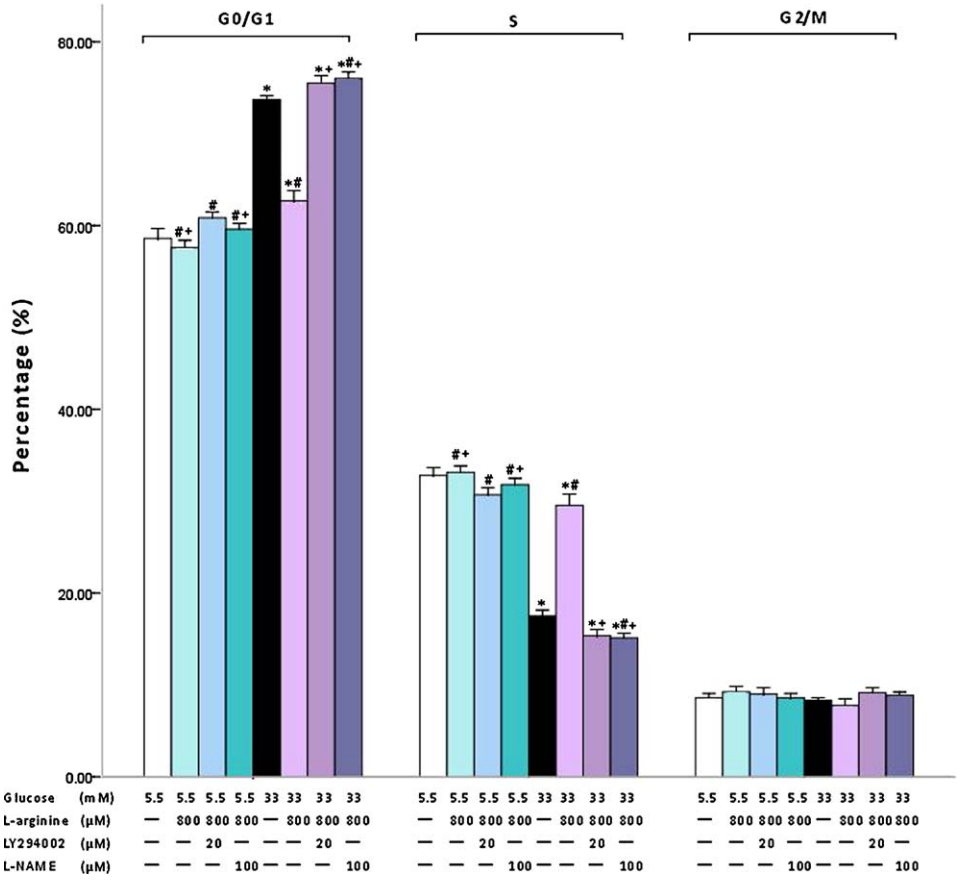


**Fig. 3 – L-arginine regulated the cell cycle and promoted cell proliferation induced by exposure to high glucose in HUVECs. HUVECs were incubated with different mediators for 24 h. Values are mean ± S.E.M for m 4 independent experiments. $^{*}p < 0.05$ compared with normal glucose group. $^{\#}p < 0.05$ compared with high glucose group. $^{+}p < 0.05$ compared with L-Arg plus high glucose group.**

and increasing cell senescence may contribute to the loss of endothelial integrity and lead to vascular complications [18]. Recent studies have provided increasing evidence that high glucose accelerates cell senescence [6,19–22], but its mechanism remains to be determined. We aimed to elucidate the possible role and mechanism of L-arginine in preventing endothelial cell senescence induced by high glucose. In this study, high glucose enhanced SA β-gal activity, depressed telomerase activity, decreased the percentage of cells in the S phase and increased that in the $G_0/G_1$ phase, inhibited Akt and eNOS activity and decreased NO production in HUVECs, thus resulting in endothelial dysfunction and cell senescence. However, L-arginine, the NO substrate, can prevent the negative effects induced by high glucose, thereby inhibiting the accelerated senescence process induced by high glucose. This effect was associated with an upregulated PI3K/Akt pathway.

Age-dependent telomere shortening could be a valid marker of biological aging [23]. Telomerase is a ribonucleoprotein that adds telomeres onto chromosome ends with its RNA moiety as a template, which is composed of 3 parts, namely, telomerase RNA (hTR), telomerase-associated protein (TP) and telomerase catalytic subunit (hTERT) [24]. With 3 functions it can add telomere DNA to the end of chromosome through its own RNA template, catalytic subunit and the auxiliary protein; maintain and balance the length of telomere sequences; and repair broken ends of chromosomes. Therefore, telomerase plays a crucial role in vascular cell senescence [8]. We found that high glucose attenuated telomerase activity, but L-arginine prevented this high glucose-induced reduction in activity by overexpressing telomerase by 194.5% as compared with the control group at 24 h.

Protein kinase Akt is a downstream effector of PI3K and is a multifunctional regulator of cell survival, cell cycle, priming of apoptosis, anti-oxidation, angiogenesis, telomerase activation, and protein synthesis, for example. Recent studies have shown that certain factors, by up-regulating PI3K/Akt pathway, promoted Akt phosphorylation, enhanced telomerase activity, made hTERT gene overexpression and emerged phosphorylation in the corresponding sites; but application PI3K inhibitors reduced telomerase activity and inhibited the phosphorylation of hTERT [25–27]. And it played an anti-aging and anti-oxidative stress by upregulating PI3K/Akt in transfected hTERT overexpression endothelial cells [28]. Therefore, telomerase becomes an important downstream in Akt pathway. In our study, we gained some similar results. High glucose inhibited Akt activity, and telomerase activity correspondingly attenuated; while L-arginine enhanced Akt activity

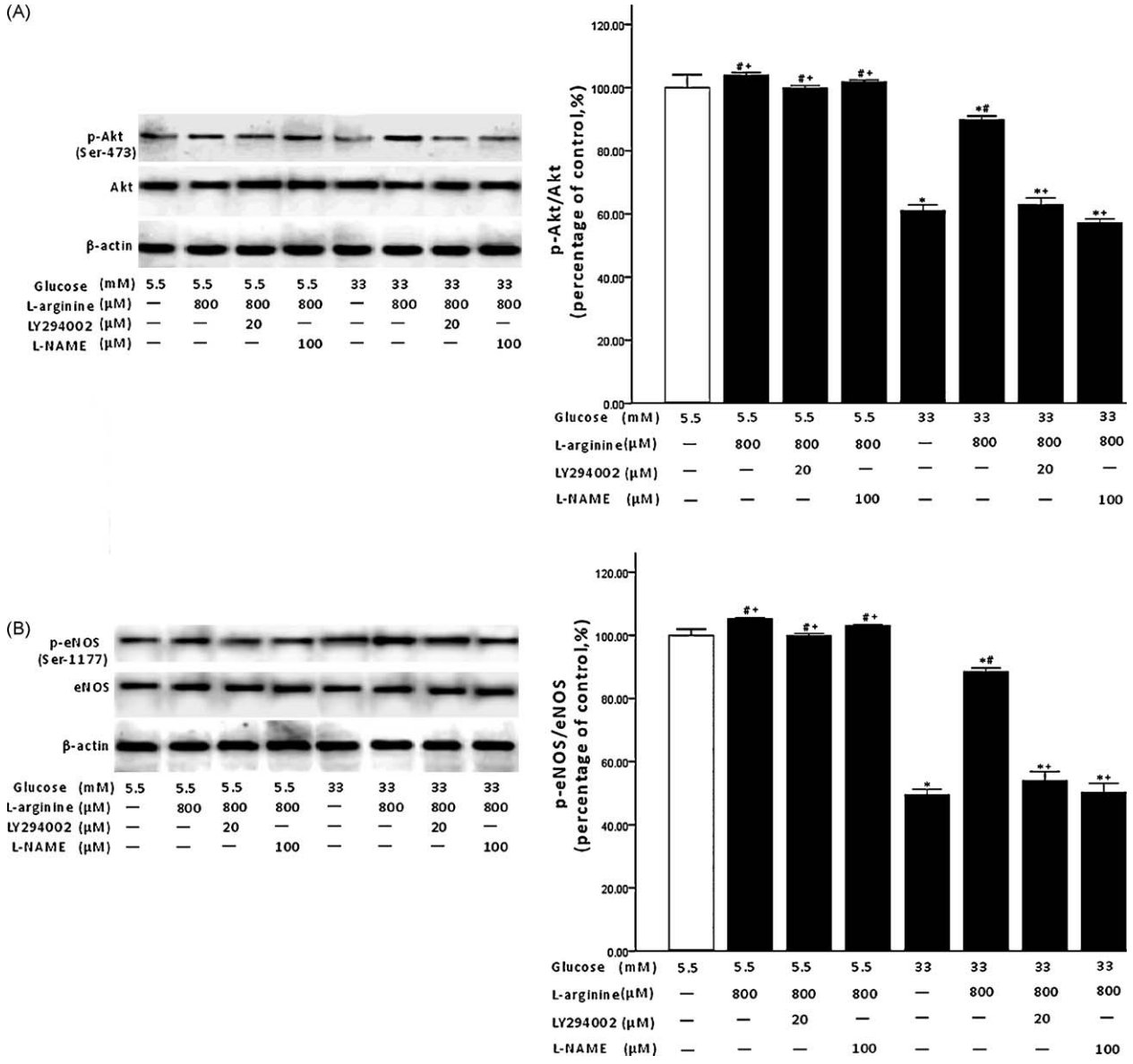


**Fig. 4 – Effect of L-arginine on PI3k/Akt/eNOS pathway under high-glucose condition in HUVECs. We used the ratio of phosphorylation to total protein expression to evaluate Akt or eNOS activity. HUVECs were incubated with different mediators for 24 h. Values are mean ± S.E.M for m 4 independent experiments. A,B $^{*}p < 0.05$ compared with normal glucose group. $^{\#}p < 0.05$ compared with high glucose group. $^{+}p < 0.05$ compared with L-Arg plus high glucose group.**

and telomerase activity correspondingly increased. However, application of LY294002 significantly attenuated telomerase activity, thus, abolished the anti-senescence effect of L-arginine.

Studies found the expression and activity of eNOS are reduced in senescent endothelial cells [20,29–34]. By inducing eNOS activity, the PI3K/Akt/eNOS pathway can provide an enhanced survival signal for the cells [15,35]. However, whether the PI3K/Akt/eNOS pathway regulates cell senescence induced by high glucose is unclear. We found that L-arginine can affect senescence induced by high glucose in HUVECs (Figs. 1–3) and could protect cells against aging by upregulating Akt (Fig. 4 A). This upregulation was accompanied by the upregulation of eNOS (Fig. 4 B). Moreover, eNOS expression was inhibited by PI3K inhibitors (Fig. 4 B). Therefore, the PI3K/Akt pathway plays a crucial role in eNOS activation. In contrast, few studies found the expression and activity of eNOS are increased in senescent cells [36–38]. It might be associated with inducing way of senescence and effect time of inducing factor. It had been reported that the expression of Akt and eNOS are reduced under long-lasting high glucose stimulation in HUVEC [15].

NO is a crucial mediator in endothelial vasodilator function. It is synthesized from the terminal guanidino nitrogen of L-arginine by NOS enzymes [36]. The bioavailability of NO is a representation of endothelial function. In the vascular system, the bioavailability of NO can be impaired by various mechanisms, including decreased NO production by

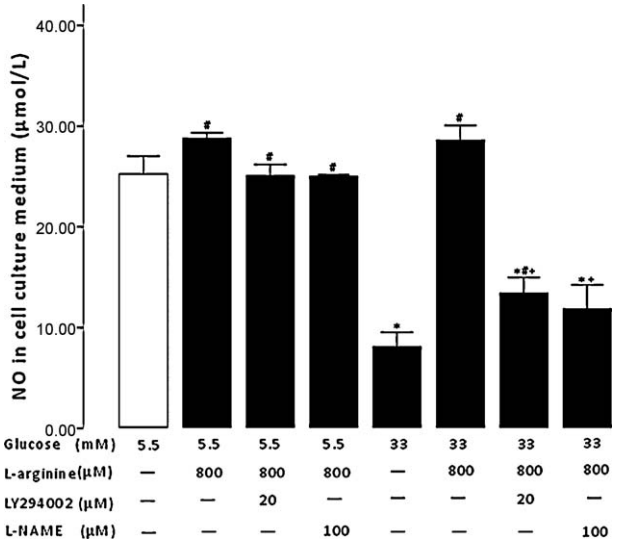


**Fig. 5 – L-arginine increased NO production induced by exposure to high glucose in HUVECs. HUVECs were incubated with 5 different mediators for 24 h. Values are mean ± S.E.M for m 4 independent experiments. $^{*}p < 0.05$ compared with normal glucose group. $^{\#}p < 0.05$ compared with high glucose group. $^{+}p < 0.05$ compared with L-Arg plus high glucose group.**

eNOS and /or enhanced NO breakdown because of increased oxidative stress [6]. In this study, high glucose inhibited NO synthesis (Fig. 5). L-arginine could rescue NO synthesis, thereby improving endothelial function and reducing the anti-senescence effect. The PI3K/Akt inhibitor LY294002 or eNOS inhibitor L-NAME could reduce NO production enhanced by L-arginine, by 79.7% or 82.0%, so the PI3K/Akt/eNOS pathway plays a role in the NO synthesis.

L-arginine is a substrate for NO synthesis. In most studies, acute and chronic administration of L-arginine improves endothelial function in animal models of hypercholesterolemia and atherosclerosis [39–42]. Recent research demonstrated that oral administration of L-arginine plus L-citrulline, alone or in combination with antioxidants for 12 weeks in rabbits fed a high-cholesterol diet reversed the progression of atherosclerosis by increasing eNOS expression and NO levels [43]. As well, oral L-arginine improved flow-mediated endothelium-dependent vasodilation of the brachial artery in a study of healthy elderly subjects; pretreatment endothelium- dependent vasodilation in these patients was abnormal [44]. In our study, we chose appropriate concentration of L-arginine (800 μM) to carry on follow study through preliminary experiment. In previous experiment, we investigated effect of four different concentration (400 μM, 800 μM, 1.6 mM, 3.2 mM) of L-arginine on high glucose accelerated endothelial cell senescence. We found anti-senescence effect of 800 μM L-arginine was better. In this study, we found that L-arginine could attenuate SA β-gal activity, enhance telomerase activity, promote cell proliferation, increase Akt and eNOS activity and accordingly increase NO production. Pretreatment with inhibitors LY294002 or L-NAME significantly increased the number of senescent cells and reduced the NO production to levels similar to those with high glucose alone. Interestingly, the effects of L-arginine on eNOS phosphorylation and NO production were also blocked by LY294002, which indicates that L-arginine-promoted eNOS phosphorylation and NO production depends on PI3K/Akt activation. These results provide strong evidence that L-arginine exerts its anti-senescent effect through the PI3K/Akt/eNOS pathway. Taken together, it is a new mechanism different from the classical role of L-arginine as the substrate of nitric oxide synthase. L-arginine might exert anti-senescence effect under high glucose condition through 2 pathways. One aspect, L-arginine might directly enhance telomerase activity to maintain the length and function of telomere by upregulation PI3K/Akt. Another aspect, L-arginine might increase NO synthesis to decrease oxidative stress by upregulation PI3K/Akt/eNOS, so endothelial function was improved and telomerase activity was indirectly enhanced. Therefore, PI3K/Akt plays an important role in the process of delaying cell senescence. But recently, Scalera F et al found that during human endothelial cell senescence the translational and posttranslational activation of Arg II contributes to accelerated senescence in the presence of therapeutically relevant concentrations of L-arginine [38]. They studied that endothelial cell senescence is accelerated in long-lasting high concentrations of L-arginine, but the mechanism of this effect has not been fully clarified. Therefore, further studies will be needed to clarify our speculation about effect and mechanism of L-arginine on endothelial cell senescence induced by high glucose.

In summary, cell senescence induced by high glucose might be an important mechanism in the pathological changes of macrovascular complications in diabetes [6,45–49]. We found that L-arginine, the NO substrate, inhibited high glucose-induced senescence of cultured HUVECs and affected the senescence process through the PI3K/Akt pathway. Further studies are required to determine the effect of L-arginine on the development of endothelial cell senescence.

## Conflicts of interest

The authors declare that they have no conflict of interest.

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