

Adiponectin attenuates the premature senescence of vascular smooth muscle cells induced by high glucose through mTOR signaling pathway

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Abstract

Objective: Cardiovascular diseases and vascular aging are common in patients with diabetes. High glucose is a major cause of vascular aging and cardiovascular diseases. Premature senescence of vascular smooth muscle cells (VSMCs) is one of the main contributors to vascular aging. Adiponectin has been demonstrated to have an anti-aging effect. The present study explored the mechanisms by which adiponectin protects VSMCs against high-glucose-induced senescence.

Methods: Senescence-associated β -galactosidase (SA- β -gal) staining was used to detect senescence cells. Western blot was used for measuring protein levels. Flow cytometry was carried out to detect the cell cycle and telomeric repeat amplification protocol (TRAP)-polymerase chain reaction (PCR) silver staining was selected to measure the telomerase activity.

Results: Premature senescence of VSMCs was induced by high glucose (30 mM) in a time-dependent manner, which was verified by an increased number of senescence cells, p21 and p53 expression, as well as the decreased proliferation index. High glucose reduced telomerase activity of VSMCs via inhibition of the AMPK/TSC2/mTOR/S6K1 pathway and activation of the PI3K/Akt/mTOR/S6K1 pathway, while adiponectin treatment significantly increased telomerase activity of VSMCs through activation of AMPK/TSC2/mTOR/S6K1 signaling and inhibition of PI3K/Akt/mTOR/S6K1 signaling.

Conclusion: Adiponectin attenuated the high-glucose-induced premature senescence of VSMCs via increasing telomerase activity of VSMCs, which was achieved by activation of AMPK/TSC2/mTOR/S6K1 signaling and inhibition of PI3K/Akt/mTOR/S6K1 signaling.

KEYWORDS

adiponectin, AMPK, mTOR, PI3K/Akt, premature senescence, vascular smooth muscle cells

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1 | INTRODUCTION

Diabetes is a major metabolic and aging-related disease. China's absolute number of diabetes cases is the highest in the world. The latest data showed that the prevalence of diabetes among adults in China is as high as 10.9% with 20.2% of these cases aged over 60 years.¹ Besides, more and more evidence has revealed that cardiovascular diseases are age-related diseases, and the aging-induced physiological and morphological changes of arteries can affect the threshold, progress, and prognosis of cardiovascular diseases.² Vascular aging contributing to the high morbidity and mortality of cardiovascular diseases is widely observed in patients with diabetes.^{3,4} Vascular smooth muscle cells (VSMCs) are important components of the vascular walls and accumulated evidence suggests that VSMC senescence plays a key role in vascular aging.⁵ For example, VSMC senescence could lead to vascular calcification and atherosclerosis.⁶ Vascular calcification was an important component of the vasculopathy widely observed in patients with type 2 diabetes.^{7,8} Hyperglycemia is a major cause of various vascular pathogenesis in patients with diabetes. However, whether adiponectin can protect VSMCs from high-glucose-concentration-induced senescence is not clear.

An important discovery in the field of aging over the past decade is the anti-aging role of rapamycin, an mTOR inhibitor.^{9,10} The mTOR plays a central role in regulating multiple cellular processes, including cell proliferation, differentiation, cell cycle, and apoptosis.^{11,12} In general, mTOR typically forms two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).¹³ PI3K/Akt/mTOR is one of the classic upstream pathways of the mTORC1 signal, which affects the phosphorylation of downstream factors, such as S6K1, and the regulation of cell functions. AMPK is another upstream pathway that affects mTOR activity by directly phosphorylating the tuberous sclerosis complex 2 (TSC2). Rapamycin is an inhibitor of mTORC1.^{14,15} Our previous studies demonstrated that the mTOR signaling pathway is involved in osteoblastic differentiation and calcification of VSMCs.¹⁶ High glucose plays a key role in vascular aging in patients with diabetic vascular complications.¹⁷ However, whether mTOR signaling contributes to VSMC senescence induced by high glucose needs to be further explored.

Adiponectin, also known as Acrp30, is secreted predominantly by differentiated adipocytes.¹⁸ Adiponectin has received substantial attention for it is widely regarded as an important factor in different kinds of metabolic processes, including insulin sensitization, lipid metabolism, and inflammation.¹⁹ Recent studies showed that adiponectin had protective effects against cardiovascular diseases.²⁰ Low serum adiponectin level was associated with thoracic aortic calcification in the elderly.²¹ Besides, the arterial adiponectin expression was increased during early stages of vascular calcification.²² Our previous study demonstrated that adiponectin treatment significantly attenuated VSMC calcification via the AMPK/mTOR/S6K1 signaling pathway.²³ Besides, adiponectin was also verified as having an anti-aging effect.²⁴ Nevertheless, whether adiponectin exerts a

protective effect on VSMC senescence via the mTOR signaling pathway under high glucose has not been reported.

In the present study, we investigated the effects of adiponectin in the premature senescence of VSMCs induced by high glucose, and further clarified the mechanisms. We found that adiponectin can inhibit the high-glucose-induced premature senescence of VSMCs via increasing the telomerase activity of VSMCs, which is achieved by inhibition of the PI3K/Akt/mTOR/S6K1 signaling pathway and activation of the AMPK/TSC2/mTOR/S6K1 signaling pathway.

2 | MATERIALS AND METHODS

2.1 | Reagents

Human recombinant adiponectin was purchased from Sigma-Aldrich (SRP4901). The senescence-associated β -galactosidase (SA- β -gal) staining kit was purchased from Beyotime Biotechnology. AICAR, Compound C, LY294002, and IGF-1 were purchased from Calbiochem. Primary antibodies for p53, p21, AMPK α , phospho-AMPK α Thr¹⁷², Akt, mTOR, phospho-mTOR Ser²⁴⁴⁸, TSC2, phospho-TSC2 Thr¹⁴⁶¹, and phospho-TSC2 Ser¹³⁸⁷ were purchased from Cell Signaling Technology. PI3K/p85, phospho-PI3K/p85, phospho-Akt Ser⁴⁷³, phospho-Akt Thr⁴⁵⁰, S6K1, and phospho-S6K1 Thr³⁸⁹ were purchased from Abcam. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnology.

2.2 | Cell culture

Human VSMCs, isolated from aortic smooth muscles of female infants, were purchased from ATCC (ATCC CRL-1999) and grown in vascular cell basal medium (ATCC). Cells were passaged every 3-4 days and cultured at 37°C in a humidified atmosphere with 5% CO₂ and premature senescence of VSMCs was induced by using 30 mM glucose for 72 hours. Cells treated with 5 mM glucose were regarded as control.

VSMCs were divided into 12 different treatment groups: (1) VSMCs were treated with 5 mM glucose as control group (Control); (2) VSMCs were treated with 5 mM glucose + 1 μ g/mL adiponectin as adiponectin control (IGlu + Adipo); (3) VSMCs were treated with 30 mM glucose as the high-glucose group (hGlu) to establish the cell premature senescence model; (4) VSMCs were treated with 30 mM glucose + 10 nM rapamycin (mTOR inhibitor) (hGlu + Ram) to verify the involvement of mTOR signaling; (5) VSMCs were treated with 30 mM glucose + 0.1 mM AICAR (AMPK activator) (hGlu + AICAR); (6) VSMCs were treated with 30 mM glucose + 20 μ M LY294002 (PI3K inhibitor) (hGlu + LY); (7) VSMCs were treated with 30 mM glucose + 1 μ g/mL adiponectin (hGlu + Adipo); (8) VSMCs were treated with 30 mM glucose + 1 μ g/mL adiponectin + 20 μ M/L Compound C (AMPK inhibitor) (hGlu + Adipo + CC); (9) VSMCs were treated with 30 mM glucose + 1 μ g/mL adiponectin + 10 nM rapamycin (hGlu + Adipo + Ram); (10) VSMCs were

treated with 30 mM glucose + 1 μ g/mL adiponectin + 0.1 mM AICAR (hGlu + Adipo + AICAR); (11) VSMCs were treated with 30 mM glucose + 1 μ g/mL adiponectin + 20 μ M LY294002 (hGlu + Adipo + LY); and (12) VSMCs were treated with 30 mM glucose + 1 μ g/mL adiponectin + 50 ng/mL IGF-1 (PI3K activator) (hGlu + Adipo + IGF-1).

2.3 | Western blot

Cells were lysated and protein concentration was measured using a BCA Protein Assay kit (Beyotime). Then, 30 μ g of total protein was separated on SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). After blocking with 5% non-fat milk for 1 hour, membranes were incubated with primary antibody overnight at 4°C and subsequently incubated with HRP-labeled secondary antibody (1:3000 dilution) for 1 hour at room temperature. Reactive proteins were detected using chemiluminescent reagents (Pierce). The relative expression level of proteins was normalized to the intensity of the GAPDH band.

2.4 | SA- β -gal staining

SA- β -gal staining was performed using a SA- β -gal staining kit (Beyotime Biotechnology) according to the manufacturer's manual. Briefly, VSMCs were fixed in β -galactosidase fixation solution (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 15 minutes and washed with PBS three times. Then the cells were stained in β -galactosidase solution (1 mg/mL 5-bromo-4-chloro-3-indolyl- β -gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl₂ in PBS) at 37°C for at least 20 hours. Cells were washed with PBS after the staining of positive cells had become visible. The SA- β -gal-stain-positive cells were counted under light microscope and the percentage of blue-stained cells was calculated.

2.5 | Cell cycle analysis

VSMCs (2×10^9) were washed twice with PBS and fixed in 75% ethanol overnight at 4°C. After washing twice in PBS, the cells were stained with 50 μ g/mL (pH 7.0) propidium iodide solution (R40432, Sigma) containing 10 mg/mL RNase and incubated, avoiding light, for 30 minutes at 37°C. The cells were then transferred to flow cytometry tubes with filters for cell cycle analysis. The proliferation index (PI) was calculated: PI = [number of (S + G2)/number of total cells] \times 100%. The percentage of PI was compared.

2.6 | Telomeric repeat amplification protocol with silver staining

Telomerase activity was analyzed using a polymerase chain reaction (PCR)-based assay kit (#NKJ15DLM) with silver staining according

to the manufacturer's instructions. Briefly, VSMCs were harvested in the provided lysis reagent and the telomerase was extracted. Then telomeric repeat amplification protocol (TRAP) was carried out with two primers (TS, 5'-AAT CCGTCGAGCAGAGTT-3' and CX, 5'-CCCTTACCCTTACCCTTACCCTAA-3'). After amplification, PCR products were resolved on a 12% nondenaturing polyacrylamide gel and vacuum transferred to PAGE membranes with silver staining. The characteristic 6-bp telomerase-specific ladder was scanned in Bioshine GelX 1650 gel imaging analysis system. The integrated optical density was determined to analyze the telomerase activity of the group.

2.7 | Statistical analysis

Data are presented as mean \pm standard deviation (mean \pm SD) and analyzed using SPSS Version 17.0. The differences between two groups were analyzed using the Student's *t*-test. Differences among three or more groups were analyzed using analysis of variance. *P* < 0.05 was considered statistically significant. All experiments were repeated at least three times and the representative experimental data are shown in the figures.

3 | RESULTS

3.1 | Adiponectin inhibits premature senescence of VSMCs induced by high glucose

First, VSMCs were treated with 5, 10, and 30 mM glucose for 36, 48, 60, 72, or 84 hours. The results showed that the cells gradually aged with the increase of glucose concentration, and when the glucose concentration was 30 mM, the cells obviously reached senescence.²⁵ Besides, the senescence of VSMCs occurred gradually with the increase of passage algebra,²³ and 30 mM glucose treatment for 72 hours obviously induced premature senescence in VSMCs. Therefore, treating VSMCs with 30 mM glucose for 72 hours was used to build a premature senescence model of VSMCs. The anti-aging effect of 0.01, 0.1, and 1 μ g/mL of adiponectin was further tested and we found that 1 μ g/mL of adiponectin was most effective in attenuating the senescence of VSMCs.

The results showed that high glucose (hGlu) induced the premature senescence of VSMCs compared with control (Control), which was confirmed by the increased level of senescence-related protein p53 and p21 as well as SA- β -gal-stain-positive cells, while the 1 μ g/mL of adiponectin (hGlu + Adipo) significantly decreased the level of p53 and p21 as well as the SA- β -gal-stain-positive cells (Figure 1). Besides, cell cycle analysis showed that the mean percentage of PI was significantly lower in hGlu-treated cells than in control cells, but it was significantly increased in VSMCs with adiponectin treatment (Figure 2).

In addition, the levels of total mTOR, p-mTOR Ser²⁴⁴⁸, and p-S6K1 Thr³⁸⁹ protein were increased significantly in high-glucose

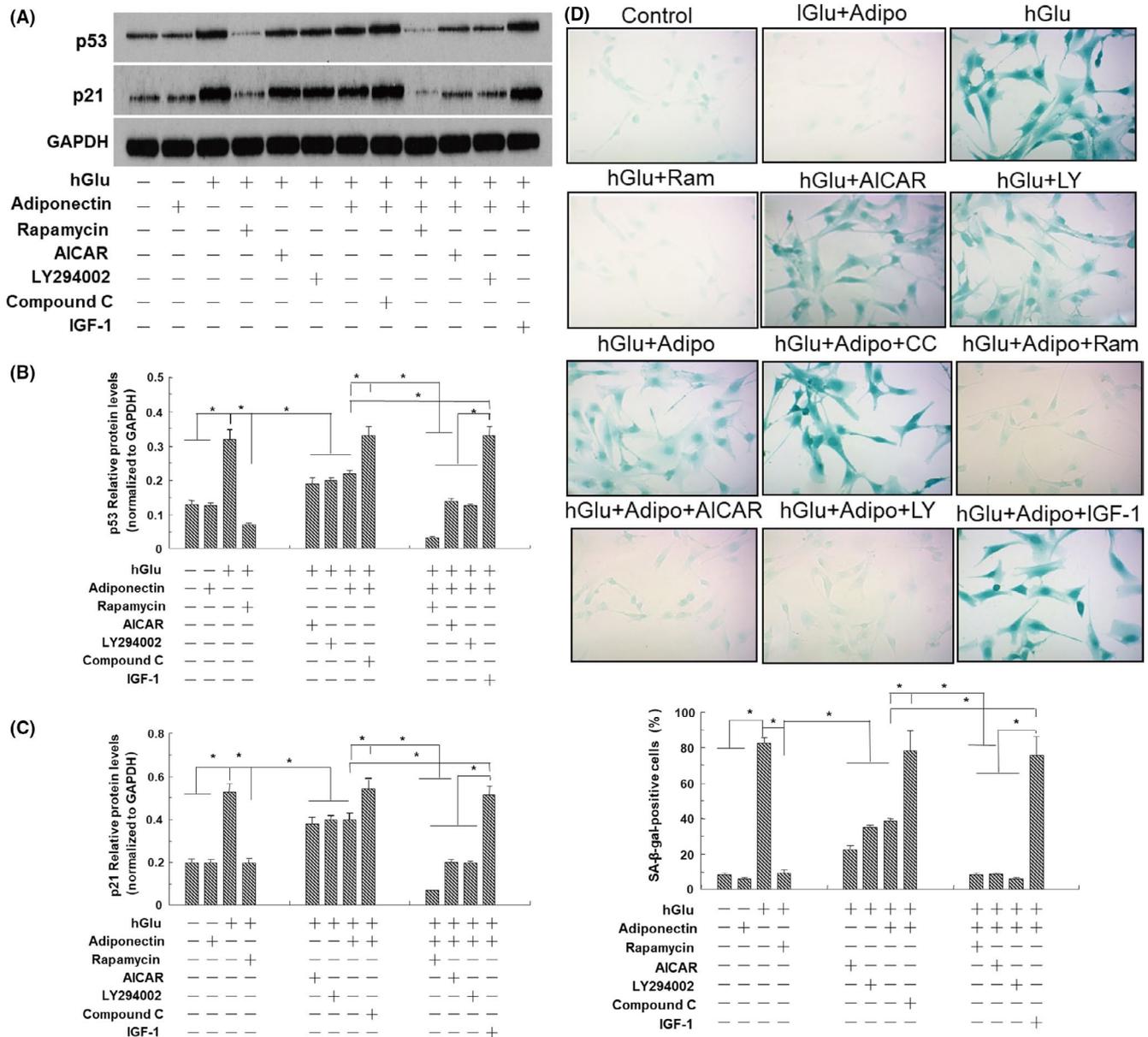


FIGURE 1 Expression of p53 and p21 and senescence-associated β -galactosidase (SA- β -gal) staining. The treatments for each group were as follows: Control: 5 mM glucose; IGlu + Adipo: 5 mM glucose + 1 μ g/mL adiponectin; hGlu: 30 mM glucose; hGlu + Ram: 30 mM glucose + 10 nM rapamycin; hGlu + AICAR: 30 mM glucose + 0.1 mM AICAR; hGlu + LY: 30 mM glucose + 20 μ M LY294002; hGlu + Adipo: 30 mM glucose + 1 μ g/mL adiponectin; hGlu + Adipo + CC: 30 mM glucose + 1 μ g/mL adiponectin + 20 μ M Compound C; hGlu + Adipo + Ram: 30 mM glucose + 1 μ g/mL adiponectin + 10 nM rapamycin; hGlu + Adipo + AICAR: 30 mM glucose + 1 μ g/mL adiponectin + 0.1 mM AICAR; hGlu + Adipo + LY: 30 mM glucose + 1 μ g/mL adiponectin + 20 μ M LY294002; hGlu + Adipo + IGF-1: 30 mM glucose + 1 μ g/mL adiponectin + 50 ng/mL IGF-1. (A) Expression of p53 and p21 protein in different groups as measured by western blot. (B,C) Semi-quantitative analysis of p53 and p21 protein levels. (D) SA- β -gal staining showed the senescence of cells in different groups and the number of SA- β -gal-stain-positive cells was calculated. N = 3. * P < 0.05, compared between two indicated groups

treated VSMCs compared to the control group. When inhibiting mTOR with rapamycin, senescence of VSMCs was reduced. Furthermore, adiponectin treatment significantly reduced the levels of these proteins compared to the high-glucose group (Figure 3). Moreover, adiponectin combined with rapamycin further attenuated VSMC senescence (Figures 1 and 2). These data indicate that adiponectin might inhibit the high-glucose-induced premature senescence of VSMCs via the mTOR-signaling pathway.

3.2 | AMPK/TSC2/MTOR signaling is involved in the regulatory effect of adiponectin in high-glucose-induced premature senescence of VSMCs

AMPK/TSC2 is one of the upstream pathways affecting mTOR activity by directly phosphorylating TSC2.²⁶ Therefore, we wondered whether AMPK/TSC2/mTOR signaling is involved in adiponectin regulating the premature senescence of VSMCs induced by high glucose. First, we found that the protein levels of p-AMPK α Thr¹⁷²

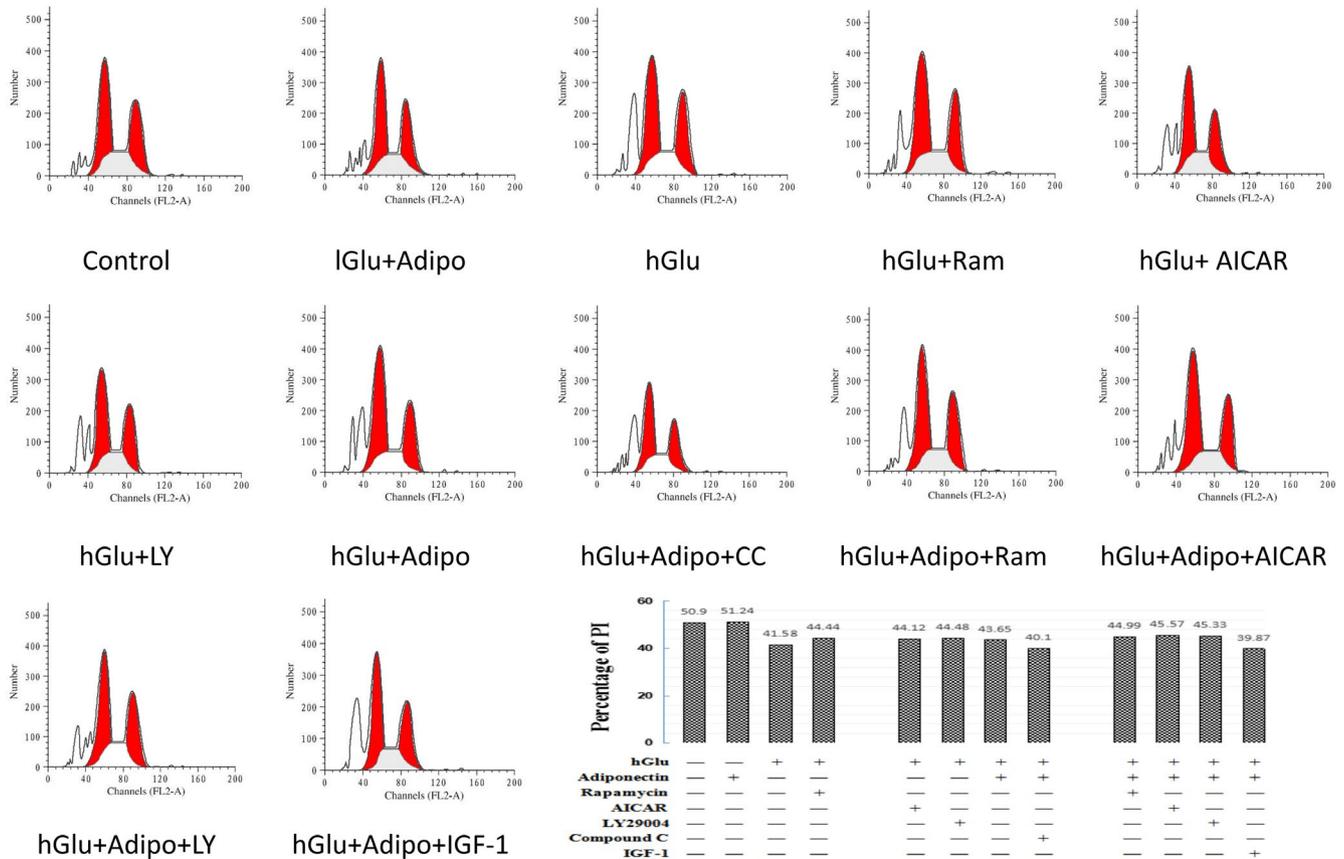


FIGURE 2 The analysis of the cell cycle. Vascular smooth muscle cells (VSMCs) were grouped as described in Figure 1. Representative images of flow cytometry were shown in different groups and the percentages of proliferation index (PI) were calculated (PI percentage: Control: 50.9%; hGlu + Adipo: 51.24%; hGlu: 41.58%; hGlu + Ram: 44.44%; hGlu + AICAR: 44.12%; hGlu + LY: 44.48%; hGlu + Adipo: 43.65%; hGlu + Adipo + CC: 40.1%; hGlu + Adipo + Ram: 44.99%; hGlu + Adipo + AICAR: 45.57%; hGlu + Adipo + LY: 45.33%; hGlu + Adipo + IGF-1: 39.87%). N = 3. * $P < 0.05$, compared between two indicated groups

and p-TSC2 Ser¹³⁸⁷ were significantly decreased in VSMCs treated with high glucose (hGlu) compared to cells treated with low glucose (Control), which were significantly increased with adiponectin treatment (hGlu + Adipo) compared to cells treated with high glucose (Figure 4).

Besides, the activation of AMPK signaling (hGlu + AICAR) significantly inhibited the levels of p53, p21, and the number of SA- β -gal-stain-positive cells (Figure 1). Moreover, the mean percentage of PI was also significantly increased in VSMCs (Figure 2). These findings suggest that high-glucose-induced premature senescence in VSMCs is mediated by the inhibition of the AMPK/TSC2-signaling pathway.

In contrast, pretreatment with the AMPK α inhibitor Compound C (hGlu + Adipo + CC), the protective effect of adiponectin on VSMCs senescence, was almost abolished (Figures 1 and 2). The levels of mTOR, p-mTOR Ser²⁴⁴⁸, and p-S6K1 Thr³⁸⁹ protein in VSMCs of the hGlu + Adipo + CC group were restored compared with the hGlu + Adipo group (Figure 3). Moreover, no changes in total AMPK α , TSC2, or p-TSC2 Thr¹⁴⁶¹ levels were observed in VSMCs with different treatments (Figure 4). These results indicate that AMPK and TSC2 protein phosphorylation, but not their protein expression, is necessary for premature senescence in high-glucose-treated VSMCs

and that adiponectin can attenuate the premature senescence of VSMCs through activation of the AMPK/TSC2/mTOR pathway.

3.3 | PI3K/AKT/MTOR signaling is involved in the regulatory effect of adiponectin in high-glucose-induced premature senescence of VSMCs

PI3K/Akt is another classic upstream pathway of mTORC1 signaling that affects the phosphorylation of downstream factors, such as S6K1, to regulate cell functions.²⁷ To investigate whether PI3K/Akt/mTOR signaling is involved in adiponectin regulating premature senescence in VSMCs, we detected the expression of PI3K p⁸⁵, p-PI3K p⁸⁵, AKT, p-Akt Ser⁴⁷³, and p-Akt Thr⁴⁵⁰.

The results showed that the levels of p-PI3K p⁸⁵, p-Akt Ser⁴⁷³, and p-Akt Thr⁴⁵⁰ protein were significantly increased in VSMCs treated with hGlu compared to the control group. These protein levels were significantly decreased by pretreatment with the PI3K inhibitor LY294002 (hGlu + LY), or by treatment with adiponectin (hGlu + Adipo), compared to cells treated with hGlu (Figure 5). At the same time, the levels of p53, p21, and number of SA- β -gal-stain-positive cells were significantly decreased in VSMCs treated

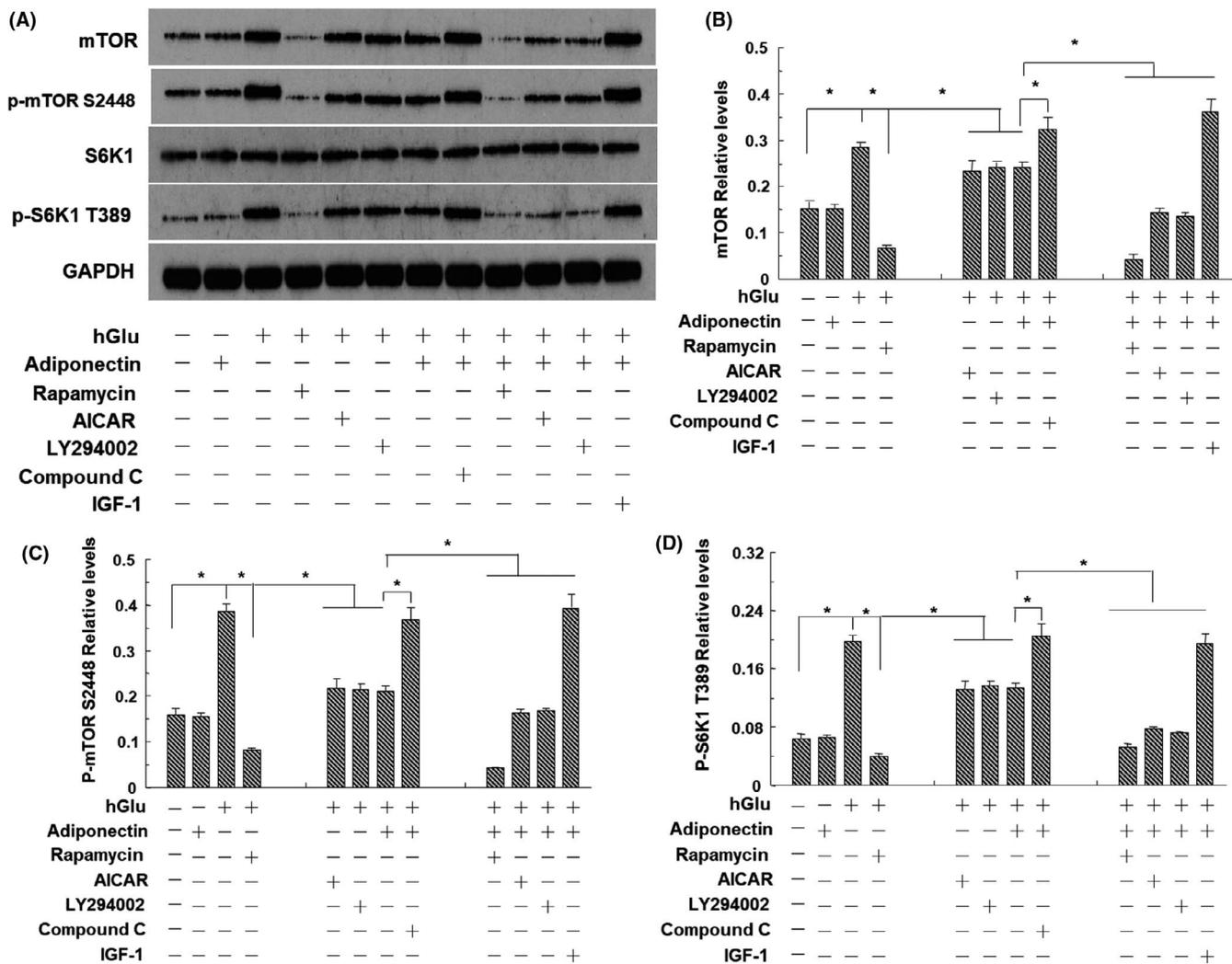


FIGURE 3 The expression of mTOR/S6K1 pathway. Vascular smooth muscle cells (VSMCs) were treated as described in Figure 1. (A) The levels of mTOR, p-mTOR Ser2448, S6K1, and p-S6K1 Thr389 protein were measured by western blot. (B) Semi-quantitative analysis of mTOR protein levels. (C) Semi-quantitative analysis of p-mTOR Ser2448 protein levels. (D) Semi-quantitative analysis of p-S6K1 Thr389 protein levels. N = 3. * $P < 0.05$, compared between two indicated groups

with hGlu + LY (Figure 1). Moreover, the mean percentage of PI was also significantly increased in VSMCs (Figure 2). These findings suggest that high-glucose-induced premature senescence in VSMCs is mediated by the activation of the PI3K/Akt signaling pathway.

By comparison, in pretreatment with the PI3K activator IGF-1 (hGlu + Adipo + IGF-1), the protective effect of adiponectin on VSMCs senescence was almost abolished (Figures 1 and 2). Besides, the levels of mTOR, p-mTOR Ser²⁴⁴⁸, and p-S6K1 Thr³⁸⁹ protein in VSMCs of the hGlu + Adipo + IGF-1 group also restored compared with the hGlu + Adipo group (Figure 3). In addition, no changes in total PI3K and Akt protein levels were observed in VSMCs among different treatment groups (Figure 5). These results indicate that PI3K and Akt phosphorylation, but not their total protein levels, are necessary for premature senescence of VSMCs induced by high glucose. Adiponectin may attenuate the premature senescence of VSMCs through inactivation of the PI3K/Akt/mTOR signaling pathway.

3.4 | Adiponectin regulates the telomerase activity of VSMCs under high glucose via AMPK/TSC2/MTOR and PI3K/AKT/MTOR pathway

Previous studies demonstrated that telomerase activity declined with in vitro aging and led to telomere shortening and cellular senescence.^{28,29} Accordingly, the telomerase activity of VSMCs treated with high glucose was much lower than that of controls (Figure 6). Nevertheless, activation of AMPK with AICAR or inhibition of PI3K with LY294002 could increase the telomerase activity of VSMCs compared with the high-glucose group (Figure 6). Meanwhile, adiponectin could also increase the telomerase activity of VSMCs induced by high glucose (Figure 6). However, when treating VSMCs with the PI3K activator IGF-1 (hGlu + Adipo + IGF-1) or AMPK α inhibitor Compound C (hGlu + Adipo + CC), the telomerase activity of VSMCs was decreased again compared with that of the hGlu + Adipo group. These results suggest that adiponectin might protect VSMCs against senescence induced by high glucose through regulating the

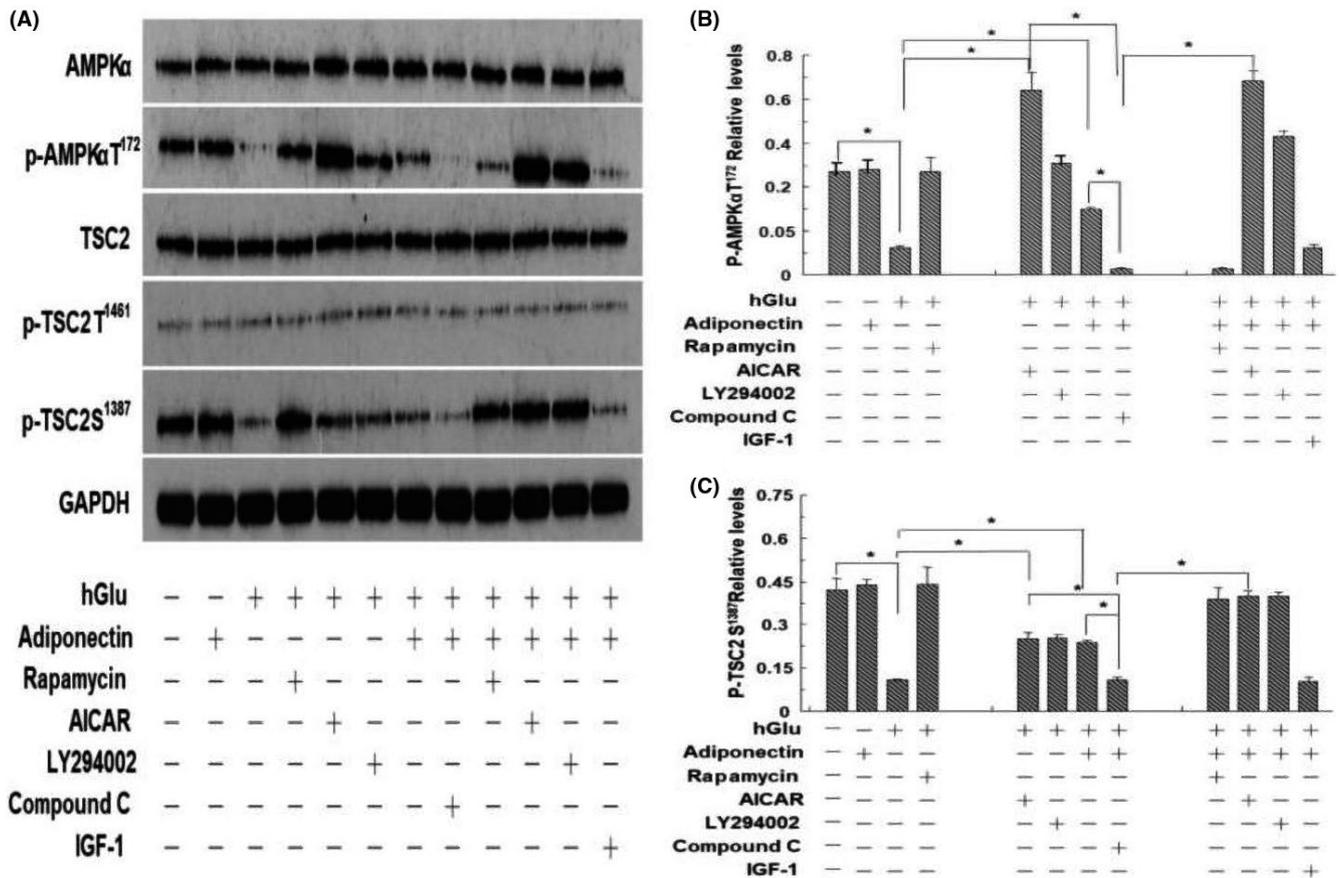


FIGURE 4 The expression of AMPK α /TSC2 signal. Vascular smooth muscle cells (VSMCs) were treated as described in Figure 1. (A) The levels of AMPK α , p-AMPK α Thr172, TSC2, p-TSC2 Thr1461, and p-TSC2 Ser1387 protein were measured by western blot. (B,C) Semi-quantitative analysis of p-AMPK α Thr172 and p-TSC2 Ser1387 protein levels, respectively. $N = 3$. * $P < 0.05$, compared between two indicated groups

telomerase activity and that the AMPK/TSC2/mTOR and PI3K/Akt/mTOR signaling pathway might be involved.

4 | DISCUSSION

In the present study, we found that high glucose decreases the telomerase activity of VSMCs and induces premature senescence of VSMCs via inhibiting the AMPK/TSC2/mTOR pathway and activating the PI3K/Akt/mTOR pathway. Moreover, adiponectin could increase the telomerase activity of VSMCs and then attenuate the senescence of VSMCs, while the AMPK/TSC2/mTOR and PI3K/Akt/mTOR pathways are involved in the process.

Vascular aging can change the onset thresholds, process, and severity of a variety of cardiovascular diseases.^{2,30} Studies have shown that high glucose is a risk factor for vascular aging in diabetes patients.⁴ Senescence of VSMCs has been found to be one of the main contributors of vascular aging.² However, whether high glucose causes the senescence of VSMCs has rarely been reported. Only our previous study has demonstrated that high glucose could induce the senescence of VSMCs.²⁵ Accordingly, the current study also demonstrated that high glucose induced significant premature senescence

in human VSMCs, as evidenced by an increase in SA- β -gal activity, upregulation of p53 and p21 expression, as well as cell cycle arrest.

Increasing evidence suggests that telomerase activity plays a key role in regulating the cell proliferation, differentiation, and replicative lifespan in normal somatic cells.^{31,32} The absence of telomerase activity in most somatic cells has been associated with telomere shortening, reduced proliferation, and senescence.^{33,34} Our previous study demonstrated that telomerase activity was reduced in the process of replicative senescence of VSMCs.²⁷ Interestingly, in the present study, we also found that telomerase activity was reduced in the process of high-glucose-induced premature senescence of VSMCs. These results suggest that telomerase activity is required for both the replicative senescence and premature senescence of VSMCs, and that telomerase may serve as a novel pharmacological target for the treatment of vascular-aging-related diseases.

The mTOR signaling is involved in a variety of cellular functions, including cell differentiation, survival, growth, metabolism, proliferation, migration, autophagy, and angiogenesis.¹¹ PI3K/Akt/mTOR is one of the classic upstream pathways of mTORC1 signaling, which affects the phosphorylation of downstream factors, such as S6K1, and their regulation of cell functions. Our recent study demonstrated that the PI3K/Akt/mTOR pathway is

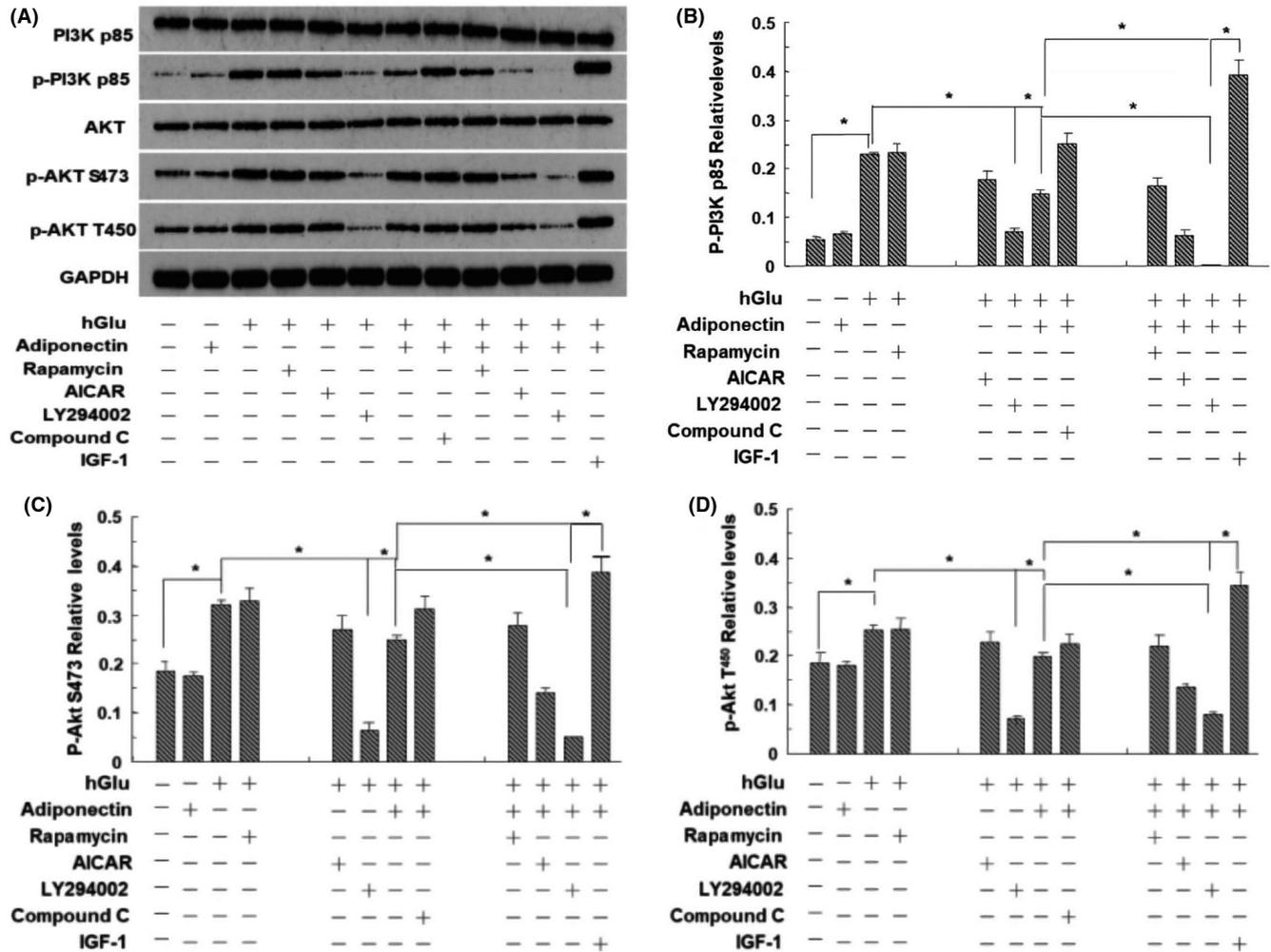


FIGURE 5 The expression of PI3K/Akt signal. Vascular smooth muscle cells (VSMCs) were treated as described in Figure 1. (A) The levels of PI3K p85, p-PI3K p85, Akt, p-Akt Ser473, and p-Akt Thr450 protein levels were measured by western blot. (B-D) Semi-quantitative analysis of p-PI3K p85, p-Akt Ser473, and p-Akt Thr450 protein levels. N = 3. *P < 0.05, compared between two indicated groups

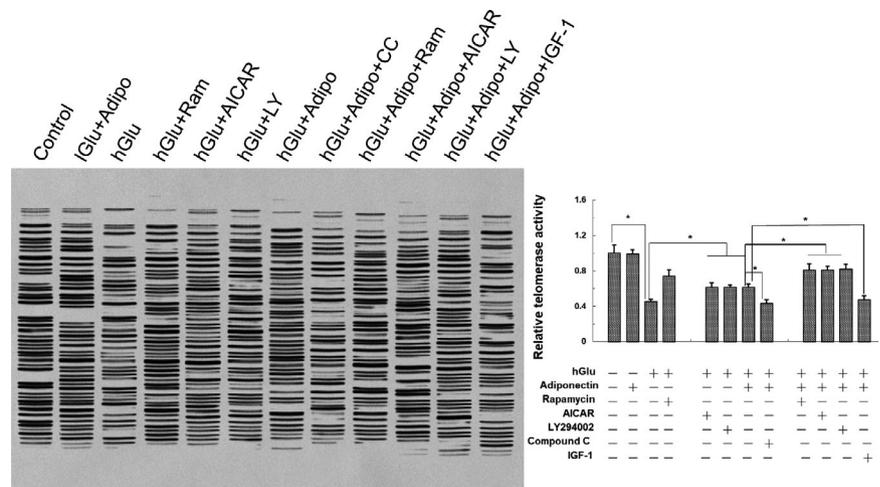


FIGURE 6 The telomerase activity of vascular smooth muscle cells (VSMCs). VSMCs were grouped as described in Figure 1. The representative images of telomerase activity detected by TRAP-PCR analysis and the relative telomerase activity were valued. N = 3. *P < 0.05, compared between two indicated groups

involved in the osteoblastic differentiation of VSMCs.³⁵ AMPK is another upstream pathway affecting mTOR activity by direct phosphorylation of TSC2.¹³ AMPK has previously been revealed to directly phosphorylate TSC2, which is a negative regulator of mTOR and subsequently inhibits mTOR activity,²³ and our

previous study demonstrated that the AMPK/TSC2/mTOR pathway regulates replicative senescence of VSMCs.²⁶ In this study, the AMPK/TSC2/mTOR signaling was inhibited and PI3K/Akt/mTOR signaling was activated during the process of high-glucose-induced premature senescence of VSMCs. Therefore, the

inhibition of AMPK/TSC2/mTOR signaling and activation of PI3K/Akt/mTOR signaling are correlated with the premature senescence of VSMCs. The activation of mTOR is mediated by phosphorylation at Ser²⁴⁸¹, Thr²⁴⁴⁶, or Ser²⁴⁴⁸ residues.³⁶ The serine/threonine kinase S6K1 is a well-known downstream target of mTORC1, which regulates S6K1 activation through its phosphorylation at Thr³⁸⁹.³⁷ Our study revealed that high glucose increased the p-S6K1 level and subsequently promoted premature senescence in VSMCs. Thus, this study provided evidence that high glucose upregulated mTOR signaling and subsequently promoted vascular aging through regulation of premature senescence of VSMCs.

Adiponectin is an adipocyte-secreted protein, abundant in human plasma, exhibiting various beneficial effects on vascular function and insulin sensitivity.^{37,38} Our previous study revealed that adiponectin could reduce VSMC calcification through the mTOR pathway.²³ VSMC calcification is a manifestation of cellular senescence and is associated with replicative senescence and stress-induced premature senescence.⁶ In the present study, the results revealed that 1 µg/mL adiponectin could significantly block the effects of high glucose in causing premature senescence of VSMCs. The mechanism study showed that adiponectin could increase the telomerase activity of VSMCs via activating AMPK/TSC2/mTOR signaling and inhibiting PI3K/Akt/mTOR signaling. These findings are in accordance with our previous study.

In conclusion, adiponectin can attenuate high-glucose-induced premature senescence in VSMCs via inhibition of PI3K/Akt/mTOR/S6K1 signaling and activation of AMPK/TSC2/mTOR/S6K1 signaling. This study suggests that adiponectin may be a potential therapeutic agent for cardiovascular and cerebrovascular diseases as well as cardiovascular complications in diabetes patients. More prospective explorations are needed to confirm this finding.

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CONFLICTS OF INTEREST

All authors of this study have declared no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors: Writing of paper. Professor Jun-Kun Zhan, You-Shuo Liu: Design, coordination, conduction. Xing-Jun Cui: Design, trial, data collection, literature review. Xiao Lin, Jia-Yu Zhong: Trial, data collection and cleansing, statistical analysis. Shuang Li: Data collection, initial statistical analysis. Yu-Qing Ni: Data analysis, second-round statistical analysis.

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