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Endothelium-specific SIRT1 overexpression inhibits hyperglycemia-induced upregulation of vascular cell senescence

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The rapidly increasing prevalence of diabetes mellitus worldwide is one of the most serious and challenging health problems in the 21st century. Mammalian sirtuin 1 (SIRT1) has been shown to decrease high-glucose-induced endothelial cell senescence *in vitro* and prevent hyperglycemia-induced vascular dysfunction. However, a role for SIRT1 in prevention of hyperglycemia-induced vascular cell senescence *in vivo* remains unclear. We used endothelium-specific SIRT1 transgenic (SIRT1-Tg) mice and wild-type (WT) mice to construct a 40-week streptozotocin (STZ)-induced diabetic mouse model. In this mode, 42.9% of wild-type (WT) mice and 38.5% of SIRT1-Tg mice were successfully established as diabetic. Forty weeks of hyperglycemia induced significant vascular cell senescence in aortas of mice, as indicated by upregulation of expression of senescence-associated markers including p53, p21 and plasminogen activator inhibitor-1 (PAI-1). However, SIRT1-Tg diabetic mice displayed dramatically decreased expression of p53, p21 and PAI-1 compared with diabetic WT mice. Moreover, manganese superoxide dismutase expression (MnSOD) was significantly downregulated in the aortas of diabetic WT mice, but was preserved in diabetic SIRT1-Tg mice. Furthermore, expression of the oxidative stress adaptor p66Shc was significantly decreased in aortas of SIRT1-Tg diabetic mice compared with WT diabetic mice. Overall, these findings suggest that SIRT1-mediated inhibition of hyperglycemia-induced vascular cell senescence is mediated at least partly through the reduction of oxidative stress.

SIRT1, hyperglycemia, vascular cell senescence

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Diabetes mellitus is a major public health problem worldwide, with estimated 285 million people affected. The prevalence of diabetes is also rapidly increasing, and is projected to rise to 366 million in 2030 [1]. Cardiovascular complications are the main cause of premature morbidity and mortality in diabetic patients [2]. As the first barrier of blood vessels, the endothelium plays an extremely important role in maintaining vessel homeostasis [3]. Hyperglycemia is considered a key factor in the development of endothelial dysfunction, which is an early feature and the main cause of diabetic vascular complications [4,5].

Aging is an independent risk factor for cardiovascular diseases. Senescent vascular cells have been detected in human atherosclerotic tissues and exhibit various functional abnormalities [6,7], suggesting that senescence of vascular

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cells contributes to the pathophysiology of age-related vascular diseases. There is also *in vivo* evidence for the occurrence of vascular cell senescence in diabetic vasculopathy [8,9]. Furthermore, high glucose-induced endothelial senescence exaggerates vascular inflammation and thrombosis in the vessels, promoting the development of diabetic-associated cardiovascular events [10].

Mammalian sirtuin 1 (SIRT1), the closest homolog of yeast sir2, functions as a NAD^+-dependent histone deacetylase. SIRT1 is known to regulate cell proliferation, apoptosis, DNA damage repair, senescence and metabolism [11], and can control longevity in response to caloric restriction (CR) in many organisms, including yeast, worms, flies and possibly mammals [12]. We previously demonstrated that overexpression of endothelium-specific SIRT1 protects against endothelial dysfunction induced by a high-fat diet and hyperglycemia [13,14]. However, it remains unknown whether SIRT1 overexpression can inhibit hyperglycemia-induced vascular cell senescence *in vivo*.

Thus, in the present study, we constructed a 40-week streptozotocin (STZ)-induced diabetic mouse model and found that endothelium-specific overexpression of SIRT1 significantly decreased expression of the senescence-associated markers p53, p21 and PAI-1 in the aortas of mice. Furthermore, the SIRT1-mediated inhibition of hyperglycemia-induced vascular cell senescence was mediated, at least in part, via a reduction in oxidative stress.

1 Materials and methods

1.1 Animals

Mice were maintained in a temperature-controlled (25°C) facility with a 12 h light/12 h dark cycle and were given free access to food and water. Endothelial cell-specific transgenic mouse lines were established by microinjecting VE-SIRT1 plasmid into fertilized C57BL/6 eggs [13]. Briefly, full-length human SIRT1 cDNA was ligated with the mouse VE-cadherin promoter. The newly cloned PBSmVE-SIRT1 plasmid contains an SV40 polyA downstream of the SIRT1 cDNA. To generate and identify transgenic mice, the PBSmVE-SIRT1 plasmid was digested with SalI/BspHI, and the fragment was purified using agarose gel electrophoresis and a DNA recovery kit (QIAGEN, Duesseldorf, Germany). Donor eggs from C57BL/6 mice (provided by the Experimental Animal Center of the CAMS/PUMC) were prepared for microinjection. The injected eggs were then transferred into the oviducts of pseudopregnant KunMing mice as foster mothers (anesthetized by tribromoethanol saturated solution, 450 μL per mouse) and allowed to develop to term. Positive transgenic mice were identified by PCR and further confirmed by Southern blot analysis. Six-week-old male endothelium-specific SIRT1-Tg mice and WT littermates, weighing 18–22 g, were used. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

1.2 Construction of the 8-week and 40-week streptozotocin (STZ)-induced diabetic mouse model

The 8-week STZ-induced diabetic mouse model was developed as previously described [14]. The 40-week STZ-induced diabetic mouse model was constructed as follows. Endothelium-specific SIRT1-Tg mice and WT littermates were randomly divided into two groups. One group was made diabetic by peritoneal injection of STZ. STZ (50 mg kg^{-1} body weight; Sigma-Aldrich, Saint Louis, Missouri, USA) was freshly dissolved in sterile citrate buffer (0.05 mol L^{-1} sodium citrate, pH 4.5) and injected into mice within 10 min of preparation. Non-diabetic controls were treated with peritoneal injection of citrate buffer alone. STZ or citrate buffer was administered for five consecutive days during the first week of the study. Blood glucose was monitored weekly using a one-touch blood glucose meter (Lifescan, Inc., Milpitas, CA, USA). Mice with a random blood glucose level >14 mmol L^{-1} for 40 consecutive weeks after STZ injection were included in the diabetic group. Mice were anesthetized by intraperitoneal injection of Avertin (400 mg kg^{-1}) and sacrificed at 40 weeks after injection. Hearts were perfused with cold 1× phosphate buffered saline (PBS). The entire aorta from the heart to the iliac bifurcation was excised and placed in cold 1× PBS. The aorta was then cleaned of fat and loose connective tissue, frozen in liquid nitrogen, and stored at −80°C.

1.3 Detection of biochemical indices

Plasma samples were obtained from 6 h fasted mice after 40 weeks of STZ injection. Total cholesterol, triglyceride, low density lipoprotein (LDL) and high density lipoprotein (HDL) levels were measured as previously described [13]. Glycated hemoglobin (HbA1c) contents of blood samples were measured with commercially available kits (Sigma-Aldrich).

1.4 Western blot analysis

Aortas were isolated and snap-frozen in liquid nitrogen. The frozen aortas were successively pulverized and dissolved in Radioimmunoprecipitation Assay (RIPA) buffer (25 mmol L^{-1} Tris-HCl pH 7.6, 150 mmol L^{-1} NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS). After complete homogenization on an ice rotator, samples were sonicated and centrifuged at 4°C. The supernatants were transferred into fresh tubes and protein concentrations were determined by the Bicinchoninic Acid (BCA) assay. Equal amounts of protein (20 μg/lane) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, Massachusetts, USA).

After being blocked, the filters were incubated with the following primary antibodies: anti-SIRT1 (07-131; Upstate, Billerica, Massachusetts, USA), anti-Shc (sc-1695; Santa Cruz Biotechnology, Santa Cruz, California, USA), anti-p53 (sc-126; Santa Cruz Biotechnology), anti-MnSOD (M99920-050; BD Transduction Laboratories, San Jose, California, USA) and anti-β-actin (A5376; Sigma). After being washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), the immune complexes were visualized with a chemiluminescence reagent. Western blots were quantified densitometrically with Quantity One software (Bio-Rad, Hercules, California, USA), and the intensity values were normalized to GAPDH.

1.5 Statistical analysis

Data are expressed as mean±SEM. Statistical analyses were performed using the two-tailed unpaired Student *t*-test or by one-way analysis of variance (ANOVA) as appropriate, to determine statistical significance between the groups. A *P*-value less than 0.05 was considered significant.

2 Results

2.1 p53 and p21 expression is unchanged in aortas of 8-week STZ-induced diabetic mice

To examine the role of SIRT1 in hyperglycemia-induced vascular cell senescence, we constructed an 8-week STZ-induced type I diabetic mouse model with WT mice and endothelium-specific SIRT1-Tg mice, as previously described [14]. p53 and p21 were expressed at similar levels in lysed aortas from control-WT, control-Tg, diabetic-WT and diabetic-Tg mice (Figure 1).

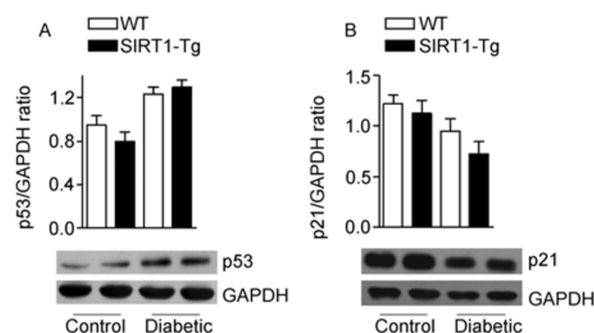

Figure 1 p53 and p21 expression levels were unchanged in lysed aortas of 8-week STZ-induced diabetic mice. Protein expression was analyzed by Western blotting. A, Bar graphs show densitometric analysis of immunoblots of p53 protein. Data are presented as the mean±SEM of p53/GAPDH expression ratio (*n*=5 per group). Immunoblots for p53 and GAPDH are representative of five independent experiments. B, Bar graphs show densitometric analysis of immunoblots of p21 protein. Data are presented as the mean±SEM of p21/GAPDH expression ratio (*n*=5 per group). Immunoblots for p21 and GAPDH are representative of five independent experiments.

2.2 Characterization of 40-week STZ-induced WT and SIRT1-Tg diabetic mice

To further elucidate the role of SIRT1 in hyperglycemia-induced vascular cell senescence, we constructed a 40-week STZ-induced diabetic mouse model. We found that 42.9% of WT mice and 38.5% of SIRT1-Tg mice were successfully established as diabetic at 40 weeks after STZ injection (Figure 2A). Analysis of blood biochemical indices demonstrated no significant changes in total cholesterol, triglycerides, HDL and LDL levels in SIRT1-Tg and WT diabetic mice compared with citrate buffer control mice (Table 1). Furthermore, there was no effect of SIRT1 overexpression on blood glucose levels at any time point (Figure 2B).

2.3 Decreased expression of senescence-associated markers in aortas of 40-week STZ-induced diabetic mice with endothelial SIRT1 overexpression

p53, p21 and PAI-1 are well established markers of senescence [15–19]. We found that expression of p53, p21 and PAI-1 was dramatically increased in aortas of 40-week STZ-induced diabetic mice compared with controls (Figure 3).

Table 1 Biochemical data from SIRT1-Tg mice and WT littermates 40 weeks after induction of diabetes using STZ (citrate buffer injection as control)[a)]

	Citrate buffer		STZ	
	WT *n*=8	Tg *n*=9	WT *n*=7	Tg *n*=5
BW (g)	30.73±2.04	31.68±2.58	28.4±2.99*	28.8±3.95*
TC (mmol L^{-1})	1.92±0.12	2.00±0.11	1.98±0.21	2.13±0.38
TG (mmol L^{-1})	0.34±0.02	0.31±0.03	0.38±0.14	0.37±0.11
HDL (mmol L^{-1})	1.89±0.04	2.01±0.12	1.88±0.22	1.86±0.21
LDL (mmol L^{-1})	0.12±0.02	0.13±0.07	0.14±0.02	0.14±0.04
Glucose (mol L^{-1})	8.02±0.63	7.48±0.56	31.04±1.39**	30.82±1.66**
HbA1c (%)	4.23±0.000	4.15±0.001	9.92±0.006**	9.66±0.008**

a) *, *P*<0.05; **, *P*<0.01 vs. corresponding controls. BW, Body weight; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HbA1c, glycated hemoglobin.

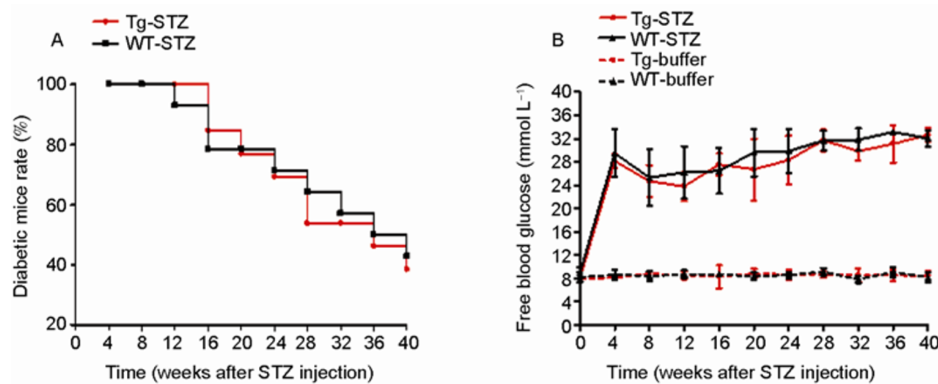

Figure 2 42.9% of WT mice and 38.5% of SIRT1-Tg mice were successfully established as diabetic 40 weeks after STZ injection and SIRT1 overexpression had no effect on blood glucose levels at any time point. A, Diabetic mice rate in WT and SIRT1-Tg mice (*n*=16 for WT, *n*=13 for SIRT1-Tg). B, Random blood glucose levels for continuous 40 weeks after injection.

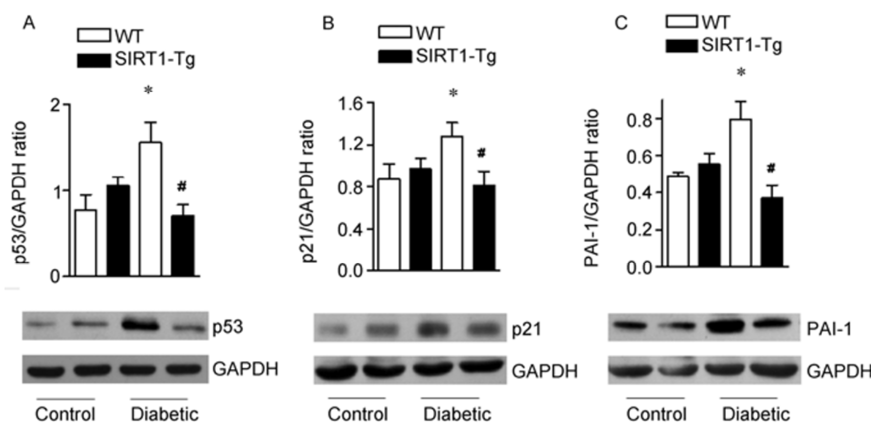

Figure 3 Endothelium-specific overexpression of SIRT1 decreases the expression levels of senescence-associated markers. Protein expression was analyzed by Western blotting. A, Bar graphs show densitometric analysis of immunoblots of p53 protein. Data are presented as the mean±SEM of p53/GAPDH expression ratio (*n*=5 per group). Immunoblots for p53 and GAPDH are representatives of five independent experiments. *, $P<0.05$ vs. WT control; #, $P<0.05$ vs. WT diabetic. B, Bar graphs show densitometric analysis of immunoblots of p21 protein. Data are presented as the mean±SEM of p21/GAPDH expression ratio (*n*=5 per group). Immunoblots for p21 and GAPDH are representative of five independent experiments. *, $P<0.05$ vs. WT control; #, $P<0.05$ vs. WT diabetic. C, Bar graphs show densitometric analysis of immunoblots of PAI-1 protein. Data are presented as the mean±SEM of PAI-1/GAPDH expression ratio (*n*=5 per group). Immunoblots for PAI-1 and GAPDH are representative of five independent experiments. *, $P<0.05$ vs. WT control; #, $P<0.05$ vs. WT diabetic.

By contrast, expression of p53, p21 and PAI-1 was significantly decreased in aortas of SIRT1-Tg diabetic mice compared with WT diabetic mice.

2.4 Endothelial SIRT1 overexpression reduced oxidative stress in aortas of 40-week STZ-induced diabetic mice

The expression of reactive oxygen species (ROS) is used as a determinant of hyperglycemia-induced endothelial dysfunction, which may contribute to vascular aging [20]. P66Shc has been shown to participate in mitochondrial ROS formation and to control the oxidative stress response [21,22]. Manganese superoxide dismutase (MnSOD) is the primary mitochondrial ROS scavenging enzyme that converts superoxide to hydrogen peroxide. As such, p66Shc and MnSOD expression levels were used to reflect oxidative stress in the mouse aortas. Diabetic mice exhibited an increase in p66Shc expression, but a decrease in MnSOD expression, in the aortas compared with controls (Figure 4). By contrast, SIRT1-Tg diabetic mice exhibited a significant decrease in p66Shc expression, but an increase in MnSOD expression, in the aortas compared with WT diabetic mice (Figure 4).

2.5 Akt phosphorylation is unchanged in aortas of 40-week STZ-induced SIRT1-Tg diabetic mice

Activation of Akt has been reported to both promote and inhibit endothelial senescence [23–25]. We found that phosphorylation of Akt was decreased in aortas of diabetic WT mice compared with controls (Figure 5). However,

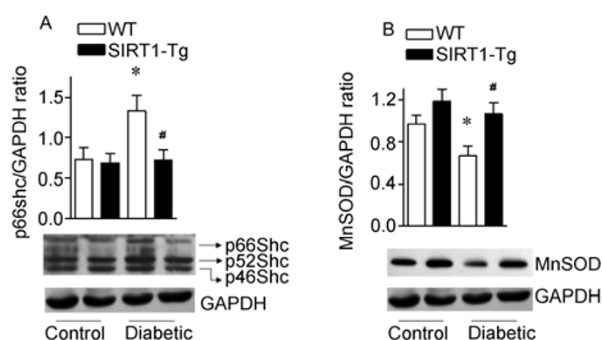

Figure 4 Endothelium-specific overexpression of SIRT1 decreases p66Shc expression and increases MnSOD expression. Protein expression was analyzed by Western blotting. A, Bar graphs show densitometric analysis of immunoblots of p66Shc protein. Data are presented as the mean±SEM of p66Shc/GAPDH expression ratio (*n*=5 per group). Immunoblots for p66Shc and GAPDH are representative of five independent experiments. *, *P*<0.05 vs. WT control; #, *P*<0.05 vs. WT diabetic. B, Bar graphs show densitometric analysis of immunoblots of MnSOD protein. Data are presented as the mean±SEM of MnSOD /GAPDH expression ratio (*n*=5 per group). Immunoblots for MnSOD and GAPDH are representative of five independent experiments. *, *P*<0.05 vs. WT control; #, *P*<0.05 vs. WT diabetic.

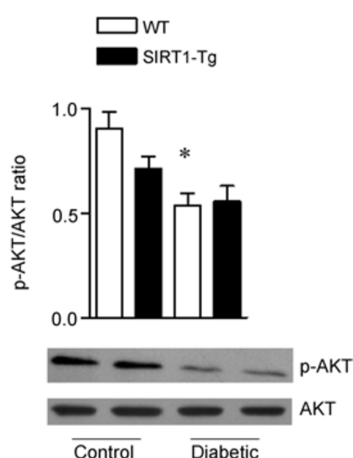

Figure 5 Endothelium-specific overexpression of SIRT1 had no effect on phosphorylation of Akt. Protein expression was analyzed by Western blotting. Bar graphs show densitometric analysis of immunoblots of total Akt and Ser473 phosphorylated Akt (Ser473 p-Akt). Data are presented as the mean±SEM of Ser473 p-Akt/Akt expression ratio (*n*=5 per group). Immunoblots for Ser473 p-Akt and Akt are representative of five independent experiments. *, *P*<0.05 vs. WT control.

there was no difference in Akt phosphorylation in aortas of SIRT1-Tg diabetic mice compared with WT diabetic mice.

3 Discussion

In the present study, we demonstrated that endothelium-specific SIRT1 overexpression inhibited the hyperglycemia-induced upregulation of the senescence-associated markers p53, p21 and PAI-1. Furthermore, SIRT1-Tg diabetic mice exhibited decreased expression of p66Shc, but increased expression of MnSOD. Thus, these data suggest that the protective role of SIRT1 against hyperglycemia-induced vascular cell senescence is mediated, at least in part, through the reduction of oxidative stress.

Accumulating evidence suggests that hyperglycemia participates in vascular cell senescence and vascular aging [26,27]. An increase of oxidative stress is associated with the majority of pathways implicated in diabetic vasculopathy, including the polyol and protein kinase C pathways [28]. Thus, these pathways may promote p53-dependent vascular cell senescence by increasing ROS levels. In support, disruption of p53 inhibits high glucose-induced endothelial senescence and dysfunction [27]. We previously reported that SIRT expression in aortas was decreased by a high-fat diet and hyperglycemia [13,14], but increased in mice with a CR diet [14,29], implying that SIRT1 might play a protective role in vascular aging. We also reported that SIRT1 overexpression significantly decreased high-glucose-induced senescence in HUVECs *in vitro* [14]. However, the p53 and p21 expression levels were unchanged in the aortas of the 8-week STZ-induced diabetic mouse model. These data suggest that changes in senescent cells may predominantly occur in the endothelium instead of the whole aortas of diabetic mice at this stage. As such, we constructed a new diabetic mouse model by extending the period of STZ treatment to 40 weeks. In this model, WT diabetic mice displayed a significant elevation of p53, p21 and PAI-1 expression levels in the aorta, which were significantly reduced in SIRT1-Tg diabetic mice. This striking induction of p53, p21 and PAI-1 expression in our 40-week diabetic mouse model may represent the development of hyperglycemia-induced vascular cell senescence, which is reversed by endothelial overexpression of SIRT1.

Oxidative stress is a major stimulus for the induction of senescence. A substantial body of evidence indicates that in endothelial cells, ROS generated from either intracellular or extracellular sources can induce or accelerate the development of senescence by acting at multiple subcellular levels [30]. Hyperglycemia plays a central role in diabetic vascular complications. Among the full spectrum of biochemical effects of high glucose, overproduction of ROS is a key determinant of hyperglycemia-induced endothelial dysfunction and contributes to vascular aging [20]. Hyperglycemia-mediated oxidative stress elicits irreversible growth arrest in endothelial cells, termed 'stress-induced premature senescence'. Endothelial cell senescence has recently been postulated as an important cause of type-2 diabetes-associated vascular aging. p66Shc has been shown to play a crucial role in the regulation of the oxidative stress response, while p66Shc deficient mice show an approximate 30% increase in life span [21]. We found that p66Shc expression was significantly decreased, while MnSOD was

dramatically increased, in aortas of both 8-week [14] and 40-week STZ-induced SIRT1-Tg diabetic mice, suggesting that the protective role of SIRT1 against oxidative stress contributes, at least in part, to its role in anti-aging. The signaling pathway of insulin/insulin-like growth factor-1/phosphatidylinositol-3 kinase/Akt is known to regulate longevity as well as resistance to oxidative stress in the nematode *Caenorhabditis elegans* [31–33]. Reduction-of-function mutations in components of this pathway have been shown to extend the lifespan in organisms ranging from yeast to mice [34,35]. However, activation of Akt has been reported to both promote and inhibit endothelial senescence [23–25]. Here we found that Akt phosphorylation was decreased in aortas of diabetic mice, similar to previous findings [24]. By contrast, there was no change in Akt phosphorylation in aortas of 40-week STZ-induced SIRT1-Tg diabetic mice compared with WT diabetic mice. Thus, these data suggest that SIRT1 may inhibit hyperglycemia-induced vascular cell senescence through reducing oxidative stress independently of Akt activity. Nevertheless, we cannot rule out the possibility that other mechanisms are involved in the anti-aging effect of SIRT1 in the endothelial cells.

In conclusion, our data provide evidence that SIRT1 significantly inhibited hyperglycemia-induced upregulation of senescence-associated markers *in vivo*. Therefore, inhibition of vascular cell senescence by activation of SIRT1 may be a potential therapeutic strategy for diabetes mellitus.

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