Repression of P66Shc Expression by SIRT1 Contributes to the Prevention of Hyperglycemia-Induced Endothelial Dysfunction

Shuang Zhou,* Hou-Zao Chen,* Yan-zhen Wan,* Qing-Jun Zhang, Yu-Sheng Wei, Shuai Huang, Jin-Jing Liu, Yun-Biao Lu, Zhu-Qin Zhang, Rui-Feng Yang, Ran Zhang, Hua Cai, De-Pei Liu, Chih-Chuan Liang

Rationale: **Inactivation of the p66Shc adaptor protein confers resistance to oxidative stress and protects mice from aging-associated vascular diseases. However, there is limited information about the negative regulating mechanisms of p66Shc expression in the vascular system.**

Objective: **In this study, we investigated the role of SIRT1, a class III histone deacetylase, in the regulation of p66Shc expression and hyperglycemia-induced endothelial dysfunction.**

Methods and Results: **Expressions of p66Shc gene transcript and protein were significantly increased by different kinds of class III histone deacetylase (sirtuin) inhibitors in human umbilical vein endothelial cells and 293A cells. Adenoviral overexpression of SIRT1 inhibited high-glucose–induced p66Shc upregulation in human umbilical vein endothelial cells. Knockdown of SIRT1 increased p66Shc expression and also increased the expression levels of plasminogen activator inhibitor-1 expression, but decreased manganese superoxide dismutase expression in high-glucose conditions. However, knockdown of p66Shc significantly reversed the effects of SIRT1 knockdown. In addition, p66Shc overexpression significantly decreased manganese superoxide dismutase expression and increased plasminogen activator inhibitor-1 expression in high-glucose conditions, which were recovered by SIRT1 overexpression. Moreover, compared to streptozotocin-induced wild-type diabetic mice, endothelium-specific SIRT1 transgenic diabetic mice had decreased p66Shc expression at both the mRNA and the protein levels, improved endothelial function, and reduced accumulation of nitrotyrosine and 8-OHdG (markers of oxidative stress). We further found that SIRT1 was able to bind to the p66Shc promoter (−508 bp to −250 bp), resulting in a decrease in the acetylation of histone H3 bound to the p66Shc promoter region.**

Conclusion: **Our findings indicate that repression of p66Shc expression by SIRT1 contributes to the protection of hyperglycemia-induced endothelial dysfunction. (*Circ Res*. 2011;109:639-648.)**

Key Words: diabetes ■ endothelial dysfunction ■ gene regulation

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Endothelial dysfunction is characterized by impaired endothelium-dependent vasorelaxation and represents an early step in the pathogenesis of several cardiovascular diseases.[1] Atherosclerotic cardiovascular complications are the leading causes of morbidity and mortality in patients with diabetes mellitus.[2] Hyperglycemia may be a key factor in the development of endothelial dysfunction. The onset and progression of complications are both delayed in diabetic patients with good glycemic control.[3,4] Therefore, protection of endothelial function against hyperglycemia-induced damages is important for preventing diabetic vascular complications.

The mammalian Shc locus encodes three Shc isoforms: p52Shc, p46Shc, and p66Shc. The p66Shc is not involved in mitogenic signals as p52Shc/p46Shc; however, it functions as a critical mediator of intracellular oxidative signals transduction.[5–8] The p66Shc participates in the production of mitochondrial reactive oxygen species by serving as a redox enzyme that oxidizes cytochrome c, thus generating proapoptotic H_2O_2 in response to specific stress signals.[9] The p66Shc-deficient mice have increased resistance to oxidative stresses and a prolonged lifespan.[7] In the vascular system, p66Shc-deficient mice are protected against age-related and

Original received February 24, 2011; revision received July 4, 2011; accepted July 14, 2011.

From the National Laboratory of Medical Molecular Biology (S.Z., H.Z.C., Y.Z.W., Q.J.Z., Y.S.W., S.H., J.J.L., Y.B.L., Z.Q.Z., R.F.Y., R.Z., D.P.L., C.C.L.), Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, P.R. China; Division of Molecular Medicine (H.C.), Departments of Anesthesiology and Medicine, Cardiovascular Research Laboratories, University of California Los Angeles (UCLA), Los Angeles, CA.

*These authors contributed equally to this work.

Correspondence to De-Pei Liu, PhD, National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 5 Dong Dan San Tiao, Beijing 100005, P.R. China. E-mail liudp@pumc.edu.cn.

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Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.111.243592

Non-standard Abbreviations and Acronyms	
Ac-H3	acetylated histone H3
ChIP	chromatin immunoprecipitation
CR	calorie restriction
H3	histone H3
HbA1c	glycated hemoglobin
HUVEC	human umbilical vein endothelial cell
MnSOD	manganese superoxide dismutase
PAI-1	plasminogen activator inhibitor-1
STZ	streptozotocin
Tg	transgenic
TSA	trichostatin A
WT	wild-type

hyperglycemia-induced endothelial dysfunction[10,11] and exhibit less atherosclerosis when fed a high-fat diet.[12] The p66Shc-deficient mice are protected against vascular, cardiac, and renal impairment attributable to major cardiovascular risk factors as recently described in a review by Cosentino et al.[13] Despite the growing body of evidence implicating the critical role of p66Shc in the pathophysiology of aging and cardiovascular diseases, there is limited information about the mechanisms that negatively regulate p66Shc expression.

Calorie restriction extends the lifespan of yeast, worms, flies, and mammals and decreases the incidence of age-associated disorders such as cardiovascular diseases and diabetes.[14,15] The beneficial effects of low caloric intake are mediated by the sirtuin family member Sir2, a conserved nicotinamide adenine dinucleotide-dependent protein deacetylase that plays a role in the extension of lifespan and chromatin remodeling associated with gene silencing.[16–17] SIRT1, the best characterized mammalian sirtuin, is involved in aging and age-related diseases.[16,17] Previous studies have established that SIRT1 plays a protective role in diabetes mellitus by participating in glucose metabolism, increasing insulin secretion and increasing insulin sensitivity.[18–20] SIRT1 is highly expressed in the vasculature and plays a critical role in the regulation of vascular function,[21–23] and SIRT1 mediates the effects of calorie restriction on endothelium-dependent vasomotor tone.[24] In addition, we have recently shown that transgenic mice that overexpress SIRT1 in the vascular endothelium have better endothelium-dependent vasodilation and fewer atherosclerotic lesions when fed a high-fat diet.[25] However, whether SIRT1 overexpression can protect against hyperglycemia-induced endothelial dysfunction remains unknown.

We therefore addressed the hypothesis that the sirtuins, specifically SIRT1, protect endothelium from hyperglycemia-induced dysfunction via downregulation of p66Shc, thereby connecting these two lifespan-controlling proteins in the vascular system.

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Methods

An expanded Materials and Methods section is available in the online Data Supplement (available at http://circres.ahajournals.org).

Cell Culture and Adenovirus Generation

Endothelial cells were freshly isolated from human umbilical cord veins as previously described[25] and cultured in M200 medium. Replication-defective adenoviral vectors expressing SIRT1 (Ad-SIRT1), SIRT1H363Y (Ad-SIRT1H363Y), or control green fluorescent protein (Ad-GFP), in addition to vectors for adenovirus-mediated knockdown of SIRT1 (Ad-SIRT1 RNAi), Ad-p66Shc RNAi, or a control RNAi vector (Ad-U6) were generated using the AdEasy Vector kit (Quantum Biotechnologies) as previously described.[25]

Induction of Diabetes in Mice by Streptozotocin Injection

Six-week-old male endothelium-specific SIRT1 transgenic (Tg) mice[25] and wild-type (WT) littermates, weighing 18 to 22 g, were randomly divided into two groups. One group was made diabetic by peritoneal injection of streptozotocin (STZ). STZ (50 mg/kg body weight) was freshly dissolved in sterile citrate buffer (0.05 mol/L sodium citrate; pH, 4.5) and injected into mice within 10 minutes of preparation. The other group was used as nondiabetic controls and received a peritoneal injection of citrate buffer alone. STZ or citrate buffer was administered for 5 consecutive days. Mice with a random blood glucose level >14 mmol/L for 3 consecutive weeks after STZ injection were specifically included in the diabetic group. Mice were anesthetized by intraperitoneal injection of Avertin (400 mg/kg) (Sigma-Aldrich, St. Louis, MO) and euthanized at 8 weeks after injection.

Isometric Tension Studies

Thoracic aortas were removed, placed in cold Krebs solution, and sectioned into 4-mm-long rings. The sectioned rings were suspended in 10-mL tissue baths. The bath was filled with Krebs solution (at 37°C, pH 7.4, with 95% O_2/5% CO_2). The preload was 0.75 g, and the rings were equilibrated for 60 minutes. The Krebs buffer solution in the tissue bath was replaced every 15 minutes. At the end of the equilibration period, the maximal force generated by addition of 3×10^{-6} mol/L phenylephrine was determined. To evaluate the endothelium-dependent relaxation, the rings were preconstricted with phenylephrine (3×10^{-6} mol/L) to obtain a stable plateau, and then a cumulative dose–response curve to acetylcholine (10^{-9} to 10^{-4} mol/L) was obtained. The nitric oxide donor sodium nitroprusside (10^{-10} to 10^{-5} mol/L) was added to test endothelium-independent relaxations.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were performed in 293A cells, human umbilical vein endothelial cells (HUVECs) or ECV304 as previously described.[26]

Statistics

Data are expressed as means±SEM. Statistical analyses were performed by two-tailed unpaired Student *t* test or by one-way analysis of variance as appropriate to determine statistical significance between the groups. $P<0.05$ was considered significant.

The authors have full access to the data and take full responsibility for the integrity. All authors have read and agreed to the manuscript as written.

Results

Inhibition of Sirtuins Induces p66Shc Expression

A previous study showed that treatment with TSA, a kind of class I and II histone deacetylase inhibitor, results in induction of p66Shc expression in cells that normally do not express p66Shc.[27] Thus, we first examined p66Shc expression after treatment with TSA and found that the expression of p66Shc was induced in 293A cells (Supplemental Figure I). To investigate whether sirtuin inhibitors could induce

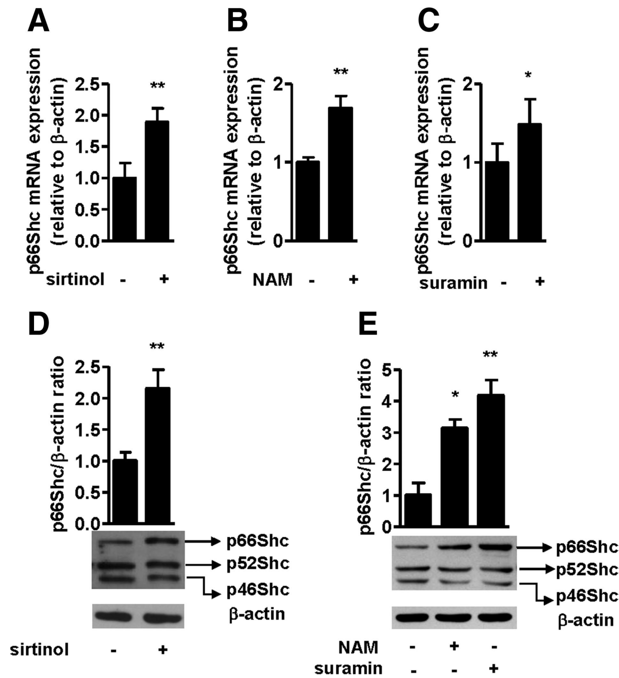

Figure 1. Class III deacetylase (sirtuin) inhibitors induce p66Shc mRNA and protein expression in human umbilical vein endothelial cells (HUVECs). HUVECs were treated with 30 μmol/L sirtinol for 24 hours (**A**), 5 mmol/L nicotinamide (NAM) for 6 hours (**B**), or 100 nmol/L suramin for 6 hours (**C**); p66Shc mRNA was analyzed by real-time polymerase chain reaction. RNA was normalized to the internal control β-actin and expressed relative to the corresponding control cells. Control cells were treated with normal medium. Data are shown as the mean$\pm$standard error of mean (SEM) for three independent experiments. *$P<0.05$, **$P<0.01$ vs corresponding control cells. **D** and **E**, HUVECs were treated with 30 μmol/L sirtinol for 24 hours (**D**), 5 mmol/L NAM, or 100 nmol/L suramin for 6 hours. The p66Shc protein was analyzed by Western blot with an anti-Shc polyclonal antibody. Bar graphs show densitometric analysis of Western blot of p66Shc protein. The densitometric quantification was normalized to β-actin. Data are shown as the mean$\pm$SEM for three independent experiments. *$P<0.05$, **$P<0.01$ vs control.

p66Shc expression, we treated 293A cells with three different kinds of sirtuin inhibitors (sirtinol, nicotinamide, and suramin) and analyzed p66Shc expressions at both the mRNA and the protein levels. All the sirtuin inhibitors significantly increased p66Shc mRNA and protein expression (Supplemental Figure II). We further treated HUVECs with sirtuin inhibitors and found that all the inhibitors also significantly increased p66Shc mRNA (Figure 1A–C), and p66Shc protein was also dramatically upregulated in HUVECs (Figure 1 D, E). These results indicated that the sirtuins are important for the inhibition of p66Shc expression.

SIRT1 Decreases High-Glucose–Induced p66Shc Expression in HUVECs

Upregulation of p66Shc is involved in hyperglycemia-induced endothelial dysfunction in diabetic mice.[11] To examine the direct effect of high glucose on p66Shc expression, we treated HUVECs with 30 mmol/L D-glucose or 30 mmol/L L-glucose. D-glucose, but not L-glucose, significantly induced p66Shc mRNA and protein expressions (Figure 2A and Supplemental Figure III) in HUVECs. However, the expression of SIRT1 in HUVECs was decreased after treated with 30 mmol/L D-glucose (Supplemental Figure IV). To further investigate whether SIRT1 inhibited high-glucose–induced p66Shc expression, HUVECs were infected with an adenovirus encoding either SIRT1 or GFP for 24 hours and then cultured with 30 mmol/L D-glucose. Time course experiments showed that overexpression of SIRT1 significantly inhibited the increase in p66Shc mRNA (Figure 2B). However, overexpression of SIRT1H363Y, which lacks the deacetylase activity, had less effect (Figure 2C and Supplemental Figure V). Likewise, high glucose also significantly induced p66Shc protein expression, and overexpression of SIRT1 significantly inhibited the increase in p66Shc protein (Figure 2D). In addition, overexpression of SIRT1 in HUVECs dramatically inhibited the increase of p66Shc mRNA expression when stimulated with 50 μmol/L oxidized low-density lipoprotein or 50 μmol/L H_2O_2 (Supplemental Figure VI).

SIRT1-Mediated Inhibition of p66Shc Expression Protects Against High-Glucose–Induced Endothelial Dysfunction

To examine the role of p66Shc in the SIRT1-mediated endothelial protection of HUVECs, adenovirus-mediated SIRT1 and p66Shc knockdown were used. SIRT1 knockdown increased p66Shc expression in high-glucose conditions (Figure 3A and 3B) and also increased the expression levels of plasminogen activator inhibitor-1, a marker of endothelial dysfunction, at both the mRNA and the protein levels. However, it decreased manganese superoxide dismutase mRNA and protein levels in HUVECs (Supplemental Figure VII). The p66Shc knockdown significantly increased the expression levels of manganese superoxide dismutase mRNA and protein, whereas its knockdown decreased plasminogen activator inhibitor-1 mRNA and protein levels in HUVECs treated with 30 mmol/L D-glucose for 24 hours (Supplemental Figure VIII). However, p66Shc knockdown significantly reversed the effects of SIRT1 knockdown under high-glucose conditions in HUVECs (Figure 3C and Supplemental Figure IX). In addition, p66Shc overexpression significantly decreased manganese superoxide dismutase protein levels and increased plasminogen activator inhibitor-1 protein levels in high-glucose conditions, which were recovered by SIRT1 overexpression (Figure 3D). These findings suggest that SIRT1-mediated inhibition of p66Shc expression protects against high-glucose–induced endothelial dysfunction in HUVECs.

Characterization of STZ-Induced WT and SIRT1-Tg Diabetic Mice

To elucidate the role of SIRT1 in diabetic vascular diseases, endothelium-specific SIRT1-Tg mice were used to construct a type 1 diabetic mouse model. STZ treatments markedly decreased body weight and increased blood glucose and HbA1c levels in SIRT1-Tg and WT mice compared with control animals injected with citrate buffer (Supplemental Table I). There were no increases in total cholesterol, triglycerides, high-density lipoprotein, and low-density lipoprotein levels in SIRT1-Tg and WT mice compared with citrate

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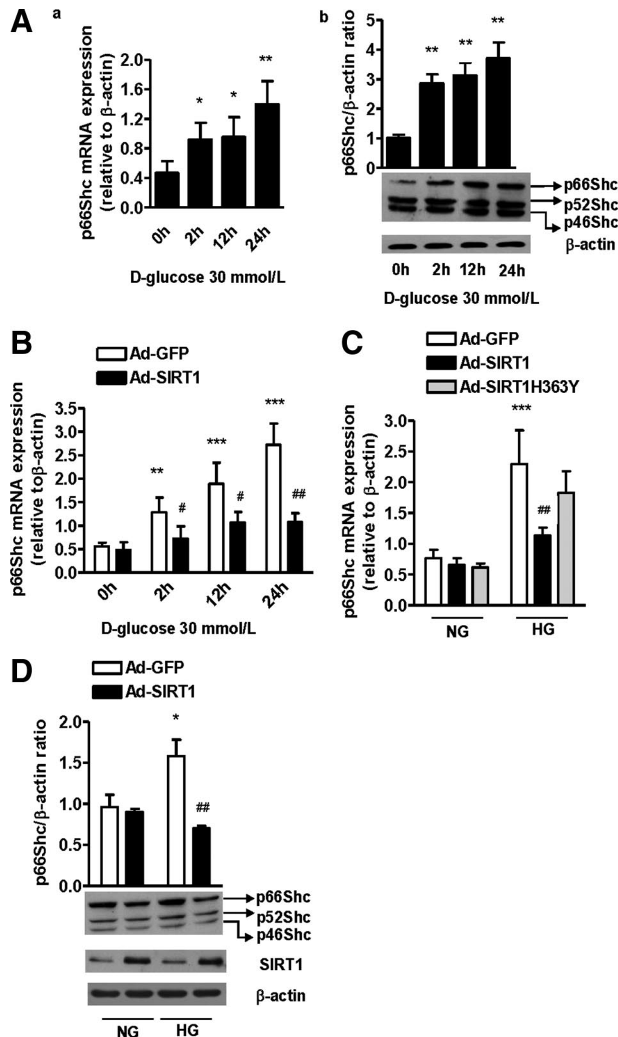

Figure 2. Overexpression of SIRT1 decreases high-glucose–induced p66Shc expression in human umbilical vein endothelial cells (HUVECs). A, High glucose (D-glucose) induces p66Shc mRNA and protein expressions in HUVECs. HUVECs were cultured in medium containing 30 mmol/L D-glucose and harvested at the indicated time points. The p66Shc mRNA (**a**) and p66Shc proteins (**b**) were analyzed by real-time polymerase chain reaction and Western blot, respectively. RNA was normalized to the internal control β-actin. Data are shown as the mean±standard error of mean (SEM) for three independent experiments. *$P<0.05$, **$P<0.01$ vs normal glucose. **B**, SIRT1 decreases high-glucose–induced p66Shc mRNA expression. HUVECs were infected with Ad-GFP or Ad-SIRT1 for 24 hours, and then cultured in medium containing high glucose (30 mmol/L D-glucose) and harvested at the indicated time points. **$P<0.01$, ***$P<0.001$ vs Ad-GFP at 0 hour, #$P<0.05$, ##$P<0.01$ vs Ad-GFP at corresponding time points. The p66Shc mRNA was analyzed by real-time polymerase chain reaction. RNA was normalized to the internal control β-actin. Data are shown as the mean±SEM for three independent experiments. **C**, HUVECs were infected with Ad-GFP, Ad-SIRT1, or Ad-SIRT1H363Y for 24 hours, and then cultured in medium containing normal (4 mmol/L D-glucose) or high (30 mmol/L D-glucose) glucose for 24 hours. The p66Shc mRNA was analyzed by real-time polymerase chain reaction. RNA was normalized to the internal control β-actin. Data are shown as the mean±SEM for three independent experiments. ***$P<0.001$ vs normal glucose Ad-GFP, ## $P<0.01$ vs high glucose Ad-GFP. D, SIRT1 decreases high-glucose–induced p66Shc protein expression. HUVECs were infected with Ad-GFP or Ad-SIRT1 for 24 hours, and then cultured in medium containing normal (5 mmol/L D-glucose) or high (30 mmol/L D-glucose) glucose for 2 hours. The p66Shc and SIRT1 proteins were analyzed by Western blot. Bar graphs show densitometric analysis of Western blot of p66Shc protein. The densitometric quantification was normalized to β-actin. Data are shown as the mean±SEM for three independent experiments. *$P<0.05$ vs normal glucose Ad-GFP, ##$P<0.01$ vs high glucose Ad-GFP. The immunoblots are representative of three independent experiments.

buffer-injected controls (Supplemental Table I). We further inspected the random glucose levels of four groups for 8 continuous weeks after STZ injection. The results showed that overexpression of SIRT1 did not affect the blood glucose levels in mice after STZ injection (Supplemental Figure X).

SIRT1-Tg Diabetic Mice Show Decreased p66Shc Expression

We first examined endogenous Sirt1 protein expression in lysed aortas from controls and diabetic WT mice. Sirt1 protein was significantly downregulated in diabetic mice after an 8-week induction compared with nondiabetic controls (Figure 4A), suggesting a potentially protective role of Sirt1 in hyperglycemia-induced endothelial dysfunction. To examine whether introduction of SIRT1 regulated p66Shc expression in vivo, we examined p66Shc at the mRNA level and found that its expression was significantly upregulated in WT mice after induction of diabetes by STZ. However, SIRT1-Tg diabetic mice displayed dramatically decreased p66Shc mRNA level compared to diabetic WT mice (Figure 4B, a). Likewise, p66Shc protein expression was upregulated in WT diabetic mice as previously reported[11] but decreased in SIRT1-Tg diabetic mice (Figure 4B, b). In addition, we examined p66Shc expression in the aortas of mice receiving calorie restriction for 1 year and found that its expression was significantly decreased compared with ad libitum-fed mice (Figure 4C). We further investigated p66Shc expression in the aortas of 18-month-old SIRT1-Tg mice and their littermate controls. The p66Shc protein expression was also significantly decreased in the aortas of SIRT1-Tg mice compared with WT mice (Figure 4D).

SIRT1-Tg Diabetic Mice Show Reduced Oxidative Stress

The p66Shc-deficient diabetic mice have reduced levels of 3-nitrotyrosine, an indicator of peroxynitrite formation.[11] In the present study, we examined nitrotyrosine residues in situ using immunohistochemistry with a polyclonal antibody against 3-nitrotyrosine. Aortas from SIRT1-Tg diabetic mice exhibited a markedly reduced immunoreactivity in both the endothelium and the medium compared with WT diabetic mice (Figure 4E, a). To further assess hyperglycemia-induced vascular oxidative stress, we measured levels of 8-OHdG, a biomarker of oxidative stress, in aortic tissue by immunohistochemistry. Hyperglycemia significantly increased 8-OHdG levels in mouse aortas; however, aortas from SIRT1-Tg diabetic mice exhibited markedly less 8-OHdG immunostaining in both the endothelium and the smooth muscle cells compared with WT diabetic mice (Figure 4E, b). Downregulation of endogenous p66Shc is known to upregulate manganese superoxide dismutase in cardiomyocytes.[28] Manganese superoxide dismutase protein was signifi-

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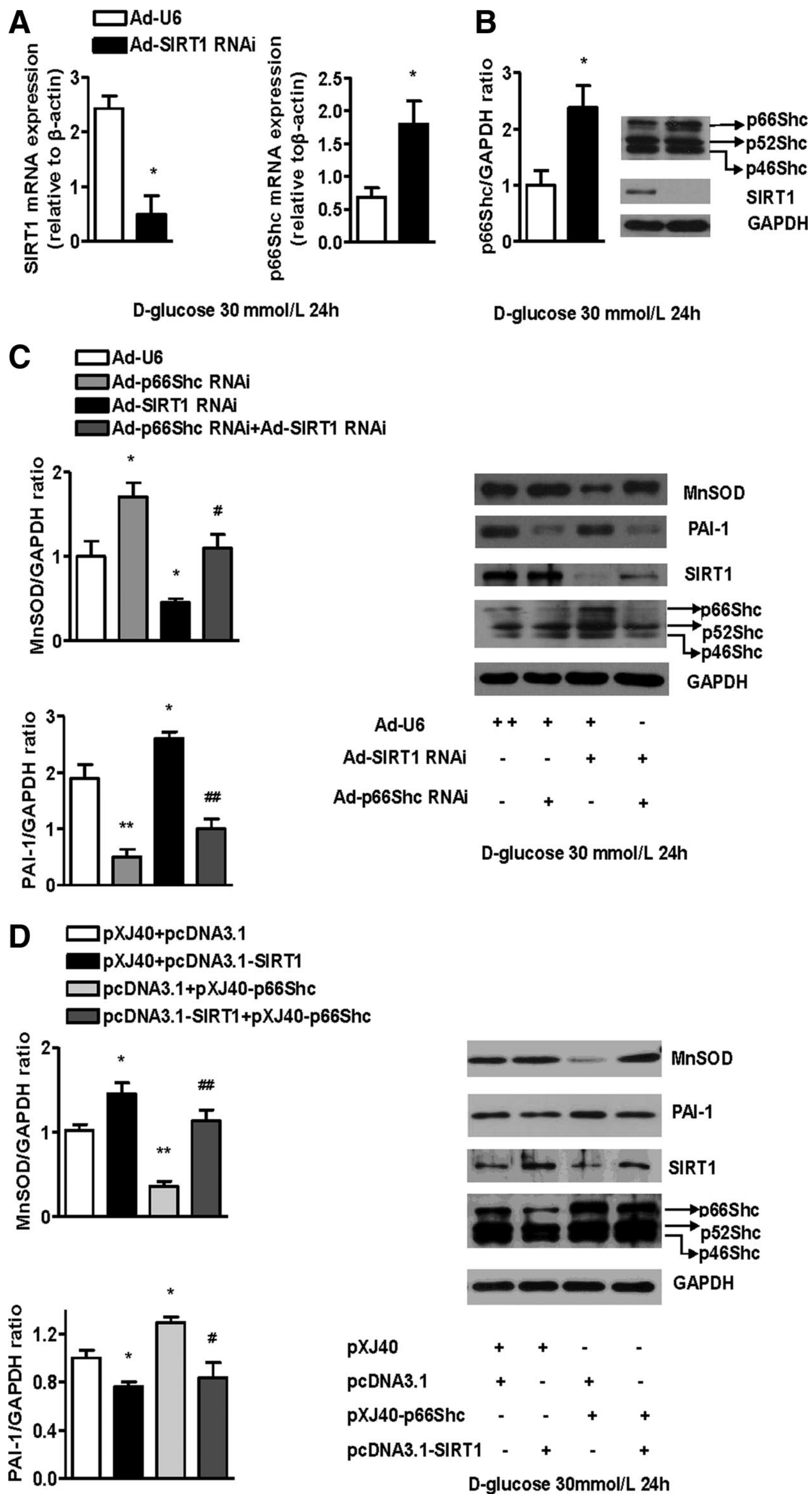

Figure 3. SIRT1 protects from high-glucose–induced endothelial dysfunction through downregulation of p66Shc in human umbilical vein endothelial cells (HUVECs). HUVECs were infected with Ad-U6 or Ad-SIRT1 RNAi for 24 hours, and then cultured in medium containing high glucose (30 mmol/L D-glucose) for 24 hours. **A**, SIRT1 and p66Shc mRNA levels were analyzed by real-time polymerase chain reaction. RNA was normalized to the internal control β-actin. Data are shown as the mean±standard error of mean (SEM) for three independent experiments. *$P<0.05$ vs Ad-U6. **B**, Bar graphs show densitometric analysis of Western blot of p66Shc protein. The densitometric quantification was normalized to GAPDH. Data are shown as the mean±SEM for three independent experiments. *$P<0.05$ vs Ad-U6. **C**, HUVECs were infected with Ad-U6 or Ad-p66Shc RNAi or Ad-SIRT1 RNAi or Ad-p66Shc RNAi and Ad-SIRT1 RNAi for 24 hours, and then cultured in medium containing high glucose (30 mmol/L D-glucose) for 24 hours. Manganese superoxide dismutase (MnSOD), plasminogen activator inhibitor-1 (PAI-1), SIRT1, and p66Shc protein levels were analyzed by Western blot. Data are shown as the mean±SEM for three independent experiments. *$P<0.05$, **$P<0.01$ vs Ad-U6. #$P<0.05$, ##$P<0.01$ vs Ad-SIRT1 RNAi. **D**, HUVECs were transfected with pcDNA3.1 and pXJ40 or pXJ40 and pcDNA3.1-SIRT1 or pcDNA3.1 and pXJ40-p66Shc or pXJ40-p66Shc and pcDNA3.1-SIRT1 for 24 hours, and then cultured in medium containing 30 mmol/L D-glucose for 24 hours. MnSOD, PAI-1, SIRT1, and p66Shc protein levels were analyzed by Western blot. Data are shown as the mean±SEM for three independent experiments. *$P<0.05$ vs pcDNA3.1 and pXJ40. #$P<0.05$ vs pcDNA3.1 and pXJ40-p66Shc.

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cantly downregulated in diabetic WT mice but was preserved in diabetic SIRT1-Tg mice (Figure 4F). Furthermore, plasminogen activator inhibitor-1 was also significantly downregulated in diabetic SIRT1-Tg mice compared with diabetic WT mice (Figure 4G).

SIRT1-Tg Diabetic Mice Display Marked Protection of Endothelial Function

Inactivation of p66Shc prevents against hyperglycemia-induced endothelial dysfunction.[11] We investigated whether overexpression of SIRT1 in the vascular endothelium could affect endothelium-dependent vasorelaxation. Isometric tension studies demonstrated that endothelium-dependent relaxation to acetylcholine was significantly impaired in WT diabetic mice, as expected (Supplemental Figure XI A). However, this effect was significantly improved in SIRT1-Tg diabetic mice (Supplemental Figure XI B). No difference was observed in vascular contractions in response to sodium nitroprusside between aortas obtained from diabetic and

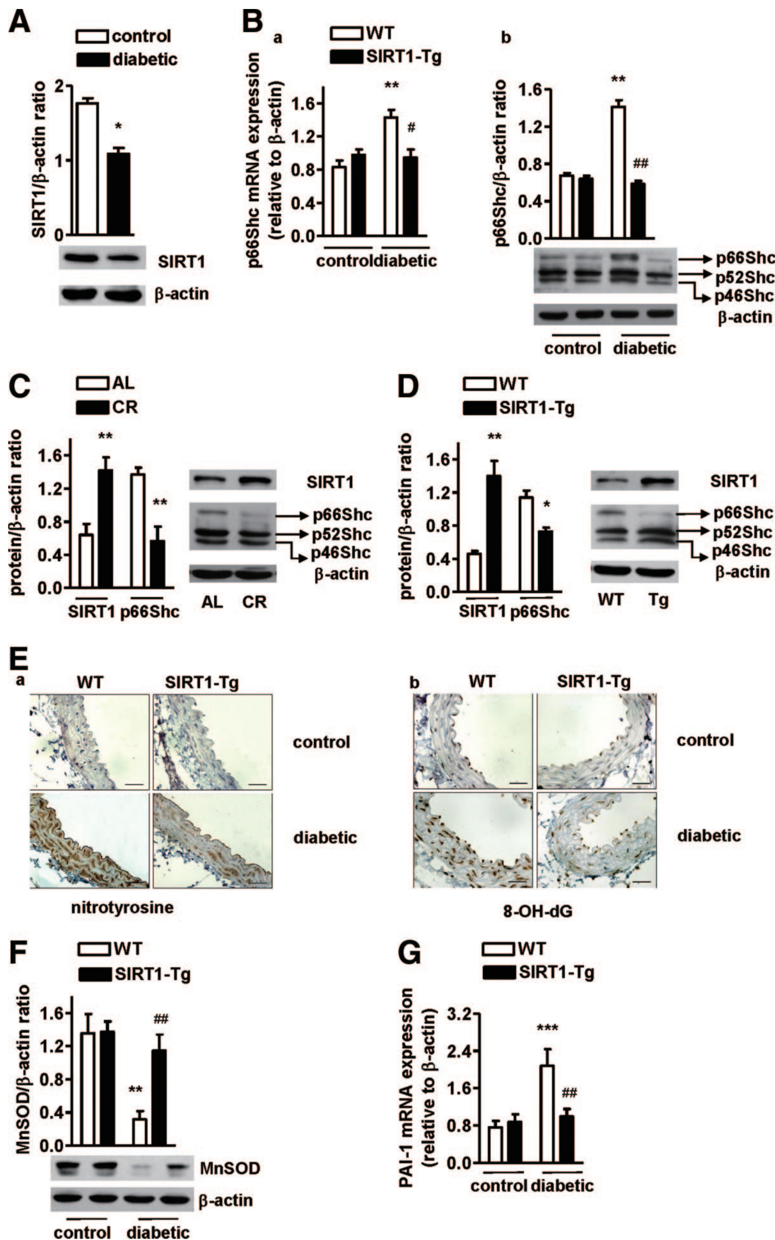

Figure 4. P66Shc expression is decreased in aortas of SIRT1 transgenic (Tg) diabetic mice. **A**, SIRT1 protein expression from aortas of control and diabetic mice. Bar graphs show densitometric analysis of SIRT1 protein immunoblots. Data are presented as the mean±standard error of mean (SEM) of SIRT1/β-actin expression ratio (n=5 in each group). *$P<0.05$ vs control wild-type (WT) mice. Immunoblots for SIRT1 and β-actin are representatives of five independent experiments. **B**, The p66Shc mRNA and protein expression in the aortas of four groups of mice. **a**, p66Shc mRNA was analyzed by real-time polymerase chain reaction. RNA was normalized to the internal control β-actin. Data are presented as the mean±SEM (n=5 in each group). **$P<0.01$ vs WT control mice; #$P<0.05$ vs WT diabetic mice. b, The p66Shc protein was analyzed by western blot. Bar graphs show densitometric analysis of immunoblots of p66Shc protein. Data are presented as the mean±SEM of p66Shc/β-actin expression ratio (n=5 in each group). **$P<0.01$ vs WT control mice, ##$P<0.01$ vs WT diabetic mice. Immunoblots for p66Shc and β-actin are representatives of five independent experiments. **C**, SIRT1 and p66Shc proteins in aortic lysates from WT male C57 mice that were fed either ad libitum (AL) or with a calorie restriction (CR) diet for 1 year. Bar graphs show densitometric analysis of immunoblots of SIRT1 and p66Shc protein. Data are presented as the mean±SEM of SIRT1/β-actin and p66Shc/β-actin expression ratio (n=5 in each group). **$P<0.01$ vs AL mice. **D**, SIRT1 and p66Shc proteins in aortic lysates from WT and SIRT1-Tg male mice that were fed AL for 18 months. Bar graphs show densitometric analysis of immunoblots of SIRT1 and p66Shc protein. Data are presented as the mean±SEM of SIRT1/β-actin and p66Shc/β-actin expression ratio (n=5 in each group). *$P<0.05$, **$P<0.01$ vs 18-month-old WT mice. **E**, SIRT1 decreases oxidative stress in the aortas of diabetic mice. a, Representative immunostaining for nitrotyrosine in the aortas of four groups of mice. Nitrotyrosine (brown staining, diaminobenzidine) was detected in both the endothelium and the medium of aortas. Hematoxylin counterstaining. Bar=50 μm. Similar immunostaining profiles were seen in six mouse aortas per group. b, Representative immunostaining for 8-OHdG in the aortas of four groups of mice. The 8-OHdG accumulation (brown staining, diamino benzidine) was detected in the nuclear of both the aortic endothelium and smooth muscle cells. Hematoxylin counterstaining. Bar=50 μm. Similar immunostaining profiles were seen in six mouse aortas per group. **F**, Manganese superoxide dismutase (MnSOD) protein in the aortas of four groups of mice was analyzed by Western blot. Bar graphs show densitometric analysis of immunoblots of MnSOD protein. Data are presented as the mean±SEM of MnSOD/β-actin expression ratio (n=5 in each group). **$P<0.01$ vs WT control mice, ##$P<0.01$ vs WT diabetic mice. Immunoblots for MnSOD and β-actin are representatives of five independent experiments. **G**, Plasminogen activator inhibitor-1 (PAI-1) mRNA in the aortas of four groups of mice was analyzed by real-time polymerase chain reaction. RNA was normalized to the internal control β-actin. Data are presented as the mean±SEM (n=3 in each group). ***$P<0.001$ vs WT control mice, ##$P<0.01$ vs WT diabetic mice.

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nondiabetic SIRT1-Tg or WT mice (Supplemental Figure XI C, D). These results indicate that upregulation of SIRT1 improves endothelial function in aortas in vivo under hyperglycemic conditions.

SIRT1 Negatively Regulates p66Shc Expression Through Epigenetic Chromatin Modification

To determine how p66Shc is regulated by SIRT1, we first examined the effect of the SIRT1 inhibitors sirtinol, NAM, and suramin on the level of acetylated histone H3 in 293A cells and found that all of these inhibitors significantly increased acetylated histone H3 (Figure 5A), providing the possibility that p66Shc transcription is regulated at the chromatin level because histone deacetylation usually leads to the inhibition of gene transcription. To explore this possibility, we performed a chromatin immunoprecipitation assay in 293A cells on a 1028-bp region of the p66Shc promoter upstream of the transcription starting site. Within this 1028-bp fragment, there was only one region (−508 bp to −250 bp) that was obviously amplified by real-time polymerase chain reaction from immunoprecipitated SIRT1 (Figure 5B), and SIRT1 bound to the same fragment of the p66Shc promoter in both HUVECs and the ECV304 cells (Figure 5C). We therefore investigated

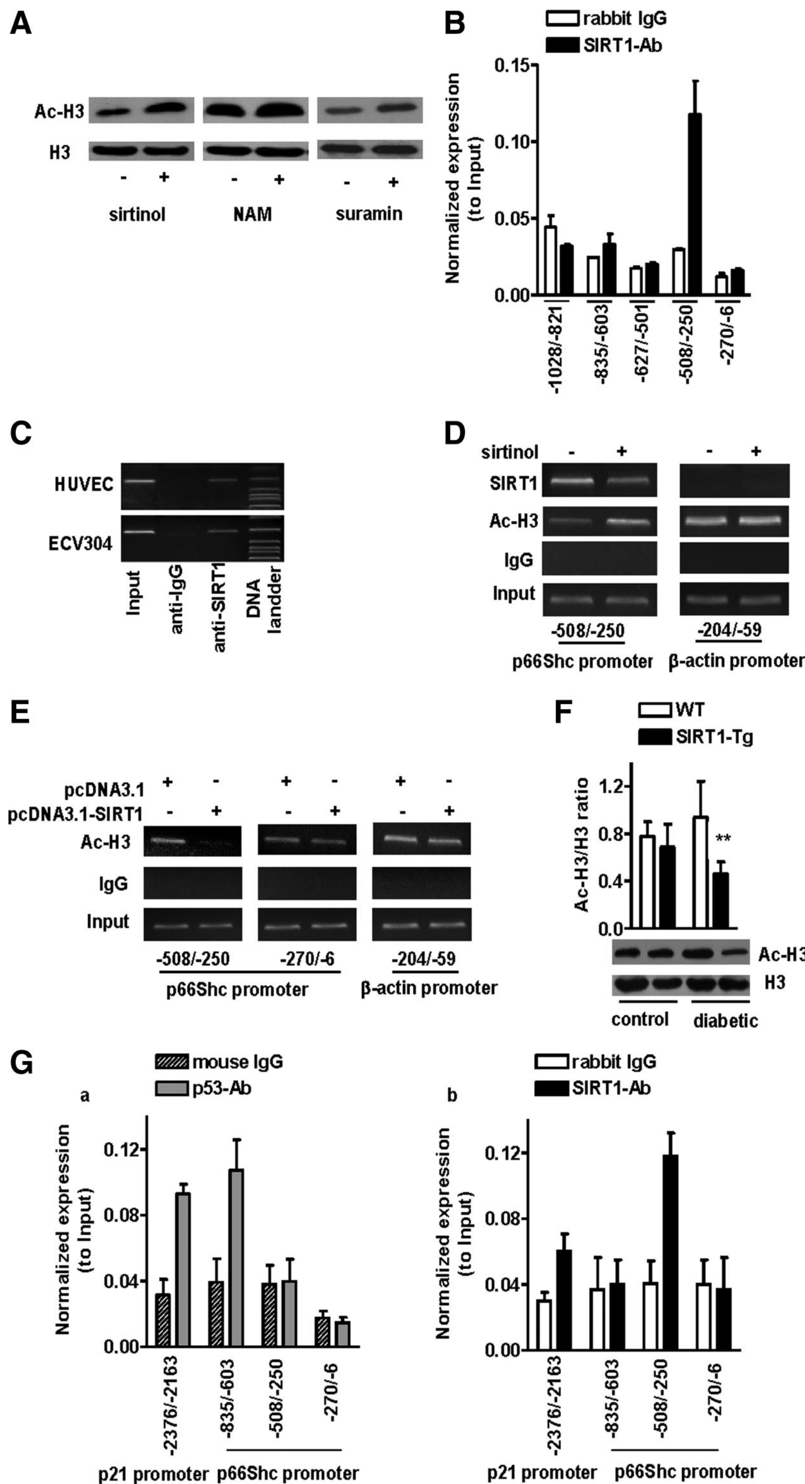

Figure 5. SIRT1 negatively regulates p66Shc expression through epigenetic chromatin modification. A, The 293A cells were treated with 30 μmol/L sirtinol for 24 hours, 20 mmol/L nicotinamide (NAM), or 100 nmol/L suramin for 6 hours. Ac-H3 protein was analyzed by Western blot. **B**, Chromatin immunoprecipitation (ChIP) assays were performed with chromatin prepared from 293A cells. Chromatin was immunoprecipitated with normal rabbit IgG or antibody against SIRT1, and precipitated genomic DNA was analyzed by real-time polymerase chain reaction using different primers for the different areas of the p66Shc promoter region. **C**, ChIP assays were performed with chromatin prepared from HUVECs or ECV304.Chromatin was immunoprecipitated with normal rabbit IgG or antibody against SIRT1, and precipitated genomic DNA was analyzed by semiquantitative polymerase chain reaction using primers for the specific p66Shc promoter region (−508 bp to −250 bp). **D**, ChIP assay on 293A cells treated with 30 μmol/L sirtinol for 24 hours. Chromatin was immunoprecipitated with normal rabbit IgG, an antibody against SIRT1, or an antibody against Ac-H3, and precipitated genomic DNA was analyzed by semiquantitative polymerase chain reaction using primers for the specific p66Shc promoter region (−508 bp to −250 bp) and the β-actin promoter region. **E**, ChIP assay on ECV304 cells transfected with pcDNA3.1 or pcDNA3.1-SIRT1. Chromatin was immunoprecipitated with normal rabbit IgG or an antibody against Ac-H3, and precipitated genomic DNA was analyzed by semiquantitative polymerase chain reaction using primers for the specific p66Shc promoter region (−508 bp to −250 bp; −270 bp to −6 bp) and the β-actin promoter region. **F**, Ac-H3 protein in the aortas of four groups of mice was analyzed by Western blot. Bar graphs show densitometric analysis of Ac-H3 immunoblots. Data are presented as the mean±standard error of mean (SEM) of Ac-H3/H3 expression ratio (n=5 in each group). ***P*<0.01 vs wild-type (WT) diabetic mice. Immunoblots for Ac-H3 and H3 are representative of five independent experiments. **G**, ChIP assays were performed with chromatin prepared from ECV304 cells. Chromatin was immunoprecipitated with normal mouse (**a**) IgG, an antibody against p53, normal rabbit (**b**) IgG, or an antibody against SIRT1, and precipitated genomic DNA was analyzed by real-time polymerase chain reaction using different primers for different sites of the p66Shc promoter region. A 213-bp region on p21 promoter (−2376 bp to −2163 bp) that has been reported to be the p53 binding site was used as a positive control.

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whether SIRT1 affected the p66Shc promoter through modification of histone H3. After treatment with sirtinol, the acetylation of histone H3 on the promoter of p66Shc (−508 bp to −250 bp) was dramatically increased, and the binding of SIRT1 to the p66Shc promoter was dramatically decreased (Figure 5D). In contrast, we detected no binding of SIRT1 and no change in acetylated histone H3 on the promoter of β-actin (−204 bp to −59 bp), which is not regulated by SIRT1 (Figure 5D). In keeping with this finding, the acetylation of histone H3 on the p66Shc promoter (−508 bp to −250 bp) was dramatically decreased by SIRT1 overexpression in ECV304 cells (Figure 5E). Furthermore, we found that the acetylated histone H3 level was significantly decreased in the aortas of SIRT1-Tg diabetic mice compared with corresponding controls (Figure 5F). The p53, a known substrate of SIRT1, was shown to impair endothelium-dependent vasorelaxation through transcriptional upregulation of p66Shc

expression.[29] Using chromatin immunoprecipitation assays, we found that the region (−835 bp to −603 bp) encompassing the putative p53 binding sequence in the human p66Shc promoter as described,[29] which is different from the SIRT1-binding region (−508 bp to −250 bp), was amplified by real-time polymerase chain reaction from immunoprecipitated p53 in ECV304 cell line (Figure 5G). We also found that SIRT1 inhibitor sirtinol did not change the binding status of p53 to the p66Shc promoter (Supplemental Figure XII).

Discussion

In the present study, we demonstrated for the first time to our knowledge that SIRT1 inhibition of p66Shc expression is protective of hyperglycemia-induced endothelial dysfunction. There are several major findings in this study. First, we demonstrated that SIRT1 inhibits high-glucose–induced p66Shc expression at both the mRNA and the protein levels and that p66Shc knockdown significantly reversed the effects of SIRT1 knockdown under high-glucose conditions in HUVECs. Second, we found that SIRT1-Tg diabetic mice have decreased p66Shc expression, which is accompanied by reduced oxidative stress and improved endothelial function. Third, we demonstrated that SIRT1 is able to bind to the p66Shc promoter, which epigenetically modifies the p66Shc promoter.

A growing body of evidence has implicated epigenetic pathways in the transcriptional control of vascular endothelial gene expression.[30] Previous studies by Ventura et al[27] demonstrated that treatment with TSA or a demethylating agent (5-aza-dC) induces p66Shc expression in cells that normally do not express p66Shc, implying that p66Shc can be reactivated through epigenetic pathways. In the present study, we found that inhibition of sirtuin deacetylases with sirtinol, NAM, or suramin significantly increased p66Shc mRNA and protein expressions in both 293A cells and HUVECs. Adenoviral overexpression of SIRT1 repressed p66Shc transcriptional level in high-glucose–induced HUVECs. Of note, adenoviral transfection of a SIRT1 mutant that lacks deacetylase activity was less effective in repressing p66Shc expression. These observations provide compelling evidence that the deacetylase activity of SIRT1 is crucial for repressing p66Shc transcription. As expected, we found that SIRT1 overexpression decreased acetylated histone H3 binding to the p66Shc promoter region, whereas inhibition of SIRT1 increased acetylated histone H3 binding to the same region (Figure 5D, E). These data suggest that SIRT1 represses p66Shc transcription at the chromatin level. Previous studies also showed that SIRT1 can affect its target gene promoter through deacetylation of histone H3 lysine 9, in agreement with our findings.[20,26,31]

The p53 binds to the putative p53 binding sequence in the p66Shc promoter and plays a positive role in p66Shc expression through transcriptional regulation,[29] and SIRT1 also can inhibit high-glucose–induced p53 transcriptional activity.[21] We found that SIRT1 bound to the p66Shc promoter (Figure 5B, C) and that the binding site of SIRT1 was different from the reported p53 binding site in the p66Shc promoter (Figure 5G). Therefore, the decreased levels of p66Shc attributable to SIRT1 could be a result of the direct inhibitory role of SIRT1 on p66Shc expression through epigenetic chromatin modification. Still, we cannot rule out the possibility that other members of the sirtuin family play a role in the regulation of p66Shc expression. The pathophysiological relationships among SIRT1, p66Shc, and p53 also remain unknown.

Hyperglycemia plays a central role in diabetic vascular complications. Among the full spectrum of biochemical effects of high glucose, overproduction of reactive oxygen species is a determinant of hyperglycemia-induced endothelial dysfunction.[32] The p66Shc plays a crucial role in the regulation of oxidative stress response both in vitro and in vivo.[7] SIRT1 also decreases the production of oxidants and increases the resistance of cells to oxidative stress.[33] In the present study, we found that SIRT1 expression is significantly downregulated, whereas p66Shc expression is significantly upregulated in the aortas of diabetic mice. Notably, both the mRNA and the protein levels of p66Shc are significantly decreased in SIRT1-Tg diabetic mice compared with WT diabetic mice. Moreover, SIRT1-Tg diabetic mice have lower levels of oxidative stress biomarkers (3-nitrotyrosine residues and 8-OHdG) and better endothelium-dependent relaxation to acetylcholine compared with WT diabetic mice, which is in line with the findings in p66Shc$^{-/-}$ diabetic mice.[11] Thus, these findings indicate that repression of p66Shc expression by SIRT1 contributes to the prevention of hyperglycemia-induced endothelial dysfunction. Considering that there are some other SIRT1 target proteins (such as FoxOs[33,34]) that play important roles in oxidative stress, it will be interesting to further elucidate the involvement of these proteins in the regulation of endothelial dysfunction by SIRT1.

Endothelial senescence plays an important role in the endothelial dysfunction of age-associated vascular diseases. Overexpression of SIRT1 or activation of SIRT1 through cilostazol inhibits sirolimus-induced, everolimus-induced, or oxidative stress-induced endothelial senescence.[35,36] Recently, Orimo et al[21] showed that the SIRT1 activator resveratrol prevents the hyperglycemia-induced vascular cell senescence and thereby protects against vascular dysfunction in vivo. Consistent with these findings, we found that SIRT1 overexpression significantly decreased high-glucose–induced senescence in HUVECs (Supplemental Figure XIII A), whereas SIRT1 inhibition increased it (Supplemental Figure XIII B). Moreover, decreased plasminogen activator inhibitor-1 expression, which is also a maker of endothelial senescence,[37] was also observed in SIRT1-Tg diabetic mice compared with WT diabetic mice, suggesting an antiaging effect of SIRT1 in the vascular system. In addition, expression of p66Shc was significantly decreased, whereas SIRT1 expression was upregulated in the aortas of mice with calorie restriction (Figure 4C) and may mediate the effects of calorie restriction on endothelium-dependent vasomotor tone.[24] Inactivation of the p66Shc gene protects against age-dependent endothelial dysfunction,[10] and decreased p66Shc protein expression was also observed in aortas of aged SIRT1-Tg mice compared with age-matched WT littermates (Figure 4D). Taken together, these data suggest that repression of p66Shc may contribute to the antiaging effect of SIRT1, leading to

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protection against endothelial dysfunction of age-associated vascular diseases.

In conclusion, our data provide evidence that SIRT1 protects blood vessels from hyperglycemia-induced endothelial dysfunction through a novel mechanism involving the downregulation of p66Shc expression. Identifying the direct link between these two lifespan-controlling proteins may provide novel therapeutic opportunities for age-associated cardiovascular diseases.

Sources of Funding

This work was supported by National Basic Research Program of China grants 2011CB503902 and 2006CB503801, National Natural Science Foundation of China grants 31021091 and 31028005; and National 863 project grant 2006AA02A406.

Disclosure

None.

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Novelty and Significance

What Is Known?

- SIRT1 is highly expressed in the vasculature and it functions as a regulator of vascular homeostasis.
- Inactivation of the p66Shc adaptor protein protects against age-related and hyperglycemia-induced endothelial dysfunction.

What New Information Does This Article Contribute?

- Inhibition of Sirtuins induces p66Shc expression.
- SIRT1 decreases high-glucose–induced p66Shc expression in human umbilical vein endothelial cells (HUVECs).
- SIRT1-transgenic (Tg) diabetic mice show decreased p66Shc expression, improved endothelial function, and reduced oxidative stress.
- SIRT1 negatively regulates p66Shc expression through epigenetic chromatin modification.

Endothelial dysfunction represents an early step in the pathogenesis of several cardiovascular diseases. In this study, we demonstrate that SIRT1, a class III histone deacetylase, negatively regulates high-glucose–induced p66Shc expression and that repression of p66Shc expression by SIRT1 contributes to the protection of hyperglycemia-induced endothelial dysfunction. Our data provide evidence that SIRT1 protects blood vessels from hyperglycemia-induced endothelial dysfunction through a novel mechanism involving the downregulation of p66Shc expression. Identifying the direct link between these two lifespan-controlling proteins may provide novel therapeutic opportunities for age-associated cardiovascular diseases.

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