Protective Role of SIRT1 in Diabetic Vascular Dysfunction

Masayuki Orimo, Tohru Minamino, Hideyuki Miyauchi, Kaoru Tateno, Sho Okada, Junji Moriya, Issei Komuro

Objective—Calorie restriction (CR) prolongs the lifespan of various species, ranging from yeasts to mice. In yeast, CR extends the lifespan by increasing the activity of silencing information regulator 2 (Sir2), an NAD⁺-dependent deacetylase. SIRT1, a mammalian homolog of Sir2, has been reported to downregulate p53 activity and thereby prolong the lifespan of cells. Although recent evidence suggests a link between SIRT1 activity and metabolic homeostasis during CR, its pathological role in human disease is not yet fully understood.

Methods and Results—Treatment of human endothelial cells with high glucose decreases SIRT1 expression and thus activates p53 by increasing its acetylation. This in turn accelerates endothelial senescence and induces functional abnormalities. Introduction of SIRT1 or disruption of p53 inhibits high glucose–induced endothelial senescence and dysfunction. Likewise, activation of Sirt1 prevents the hyperglycemia-induced vascular cell senescence and thereby protects against vascular dysfunction in mice with diabetes.

Conclusions—These findings represent a novel mechanism of vascular cell senescence induced by hyperglycemia and suggest a protective role of SIRT1 in the pathogenesis of diabetic vasculopathy. (Arterioscler Thromb Vasc Biol. 2009; 29:00-00.)

Key Words: cellular senescence • p53 • diabetes

The NAD⁺-dependent histone deacetylase Sir2 induces longevity in yeast in response to calorie restriction signals. SIRT1, a mammalian homologue of Sir2 and a member of the Sir2 family called sirtuins, has been shown to target p53, Ku70, and the forkhead transcription factors for deacetylation, thereby regulating stress responses, apoptosis, and cellular senescence. Acetylation of p53 is known to be crucial for its stabilization and transcriptional activation. Accumulating evidence suggests that SIRT1 also modulates the metabolism of glucose and fat by interacting with peroxisome proliferator-activated receptor (PPAR) γ through nuclear receptor corepressor to repress adipogenesis, modifying PPAR γ coactivator-1α to regulate hepatic glucose homeostasis1•12 and regulating insulin secretion levels as well as insulin sensitivity. Treatment with the sirtuin activator resveratrol has been shown to improve diet-induced obesity and insulin resistance and delay age-related deterioration including increased arterial stiffness. Moreover, Sirt1 has been reported to control endothelial angiogenic functions during postnatal vascular growth. However, it remains unclear whether SIRT1 is involved in the pathogenesis of diabetes and its complications including diabetic vasculopathy.

Vascular cells have a finite lifespan when cultured and eventually undergo senescence. Many of the changes seen in senescent vascular cells are consistent with those that occur in age-related vascular diseases. Moreover, senescent vascular cells have been detected in human atherosclerotic tissues and exhibit various functional abnormalities, suggesting that senescence of vascular 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. Given that CR augments SIRT1 activity, hyperglycemia might induce vascular cell senescence by reducing SIRT1 activity and thereby contribute to the development of diabetic vasculopathy. In the present study we show a novel mechanism of vascular cell senescence induced by hyperglycemia. Hyperglycemia decreases SIRT1 expression and thus activates p53 by increasing its acetylation. Activation of SIRT1 prevents the hyperglycemia-induced vascular cell senescence and thereby protects against vascular dysfunction in mice with diabetes. These results suggest a protective role of SIRT1 in the pathogenesis of diabetic vasculopathy.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells were purchased from Bio Whittaker (Walkersville, Md) and cultured according to the manufacturer’s instructions. We defined senescent cells as the cultures that do not increase for 2 weeks at subconfluent and confirmed with...
senescence-associated β-galactosidase activity assay. Senescence-associated β-galactosidase staining was performed as described.

**Retroviral Infection**

pBabe (a gift from Dr C.W. Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) was used for generating retroviruses. pBabe SIRT1 was a kind gift from Dr T. Kouzarides, Wellcome Trust, Cambridge, UK. We constructed the pBabe-based vector expressing E6 (pBabe E6). Details of the construct are available on request. Retroviral stocks were generated by transient transfection of packaging cell line and stored at −80°C until use. Human endothelial cells (passage 4 to 6) were plated at 5×10³ cells per 100-mm-diameter dish 24 hours before infections. For infections, the culture medium was replaced by retroviral stocks supplemented with 8 μg/mL polybrene (Sigma). Forty-eight hours after infections, the infected cell populations were selected by culture in 0.8 μg/mL puromycin for 4 days (pBabe-based vectors). After selection, 1 to 3×10⁵ cells were seeded onto 100-mm-diameter dish.

**Western Blot Analysis and Antibodies**

Whole-cell lysates (30 μg) were resolved by SDS polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto a polyvinylidene difluoride membrane (Millipore) and incubated with the first antibody followed by an anti-rabbit immunoglobulin G–horseradish peroxidase antibody or anti-mouse immunoglobulin G–horseradish peroxidase antibody (Jackson). Specific proteins were detected using enhanced chemiluminescence (Amersham). The first antibodies used for Western blotting are as follows: antibodies to p53, ICAM-1, actin, and tubulin (Santa Cruz); anti-p21 antibody (Oncogene); antiacetylated p53 antibody (Cell Signaling); anti-SIRT1 antibody (Upstate Biotechnology).

**Northern Blot Analysis**

Total RNA (30 μg) was extracted using RNA zol B (Tel Test) according to the manufacturer’s instructions, separated on a formaldehyde denaturing gel and transferred to a nylon membrane (Amerham). The blot was then hybridized with radiolabeled cDNA probes for p21 using the Quickhyb hybridization solution (Stratagene) according to the manufacturer’s instructions.

**Luciferase Assay**

The reporter gene plasmid (1 μg) was transfected into endothelial cells in medium containing various glucose concentrations. In some experiments, cells were infected with retroviral vectors 24 hours before luciferase assay. The control vector encoding p53 (Promega) according to the manufacturer’s instructions. The plasmid pG13-Luc containing the p53-binding sequence was a gift from Dr T. Kouzarides, Wellcome Institute, Cambridge, UK. We constructed the pBabe-based vector expressing E6 (pBabe E6). Details of the construct are available on request. Retroviral stocks were generated by transient transfection of packaging cell line and stored at −80°C until use. Human endothelial cells (passage 4 to 6) were plated at 5×10³ cells per 100-mm-diameter dish 24 hours before infections. For infections, the culture medium was replaced by retroviral stocks supplemented with 8 μg/mL polybrene (Sigma). Forty-eight hours after infections, the infected cell populations were selected by culture in 0.8 μg/mL puromycin for 4 days (pBabe-based vectors). After selection, 1 to 3×10⁵ cells were seeded onto 100-mm-diameter dish.

**Histology**

For immunohistochemistry, the frozen sections (6 μm) were treated with 0.3% hydrogen peroxide in methanol for 20 minutes, preincubated with 5% goat serum and then treated with anti-Sirt antibody (Upstate Biotechnology), anti-p53 antibody, or anti–ICAM-1 antibody (Santa Cruz) overnight at 4°C. Next, the sections were incubated with a biotinylated goat secondary antibody, treated with the avidin-biotin complex (Elite ABC kit, Vector) and stained with diaminobenzidine tetrahydrochloride and hydrogen peroxide. Senescence-associated β-galactosidase activity assay was performed as described previously.

**Statistical Analysis**

Data were shown as mean±SEM. Multiple group comparison was performed by 1-way ANOVA followed by the Bonferroni procedure for comparison of means. Comparisons between 2 groups were analyzed by 2-way ANOVA. Values of P<0.05 were considered statistically significant.

**Results**

**Treatment With High Glucose Accelerates Endothelial Cell Senescence**

To examine the effects of high glucose on the lifespan of vascular cells, human vascular endothelial cells were passaged in medium containing various concentrations of glucose (100, 150, and 400 mg/dL) until senescence occurred. The osmotic pressure of each medium was adjusted to that of the high-glucose (400 mg/dL) medium by the addition of mannitol. Exposure to a very high concentration of glucose (400 mg/dL) decreased the lifespan of human endothelial cells, and the effects of glucose were dose-dependent (Figure 1A). SIRT1 expression was decreased and acetylated p53 was increased in human endothelial cells by culture in high-glucose medium (Figure 1B and supplemental Figure I, A). SIRT1 expression was decreased and acetylated p53 was increased in human endothelial cells by culture in high-glucose medium (Figure 1B and supplemental Figure I, B). Human endothelial cells were cultured in medium containing various concentrations of glucose for 24 hours and harvested to examine SIRT1, acetylated p53 (Ac-p53), p53, p21, and ICAM-1 levels by Western blot analysis. C, Transcriptional activity of p53 was examined by luciferase assay in endothelial cells exposed to glucose at the indicated concentration. *P<0.05 vs 100 mg/dL glucose; **P<0.01 vs 0 mg/dL glucose; #P<0.05 vs 100 mg/dL glucose. Error bars indicate SEM; n = 4.
in high-glucose medium also upregulated the expression of intracellular adhesion molecule-1 (ICAM-1), a crucial receptor that mediates cell-cell interactions and plays a critical role in the development of atherosclerosis (Figure 1B and supplemental Figure I).

**SIRT1 Inhibits High Glucose-Induced Endothelial Cell Senescence**

To investigate whether SIRT1 was involved in high glucose-induced senescence, we examined the effect of increased SIRT1 expression on activation of p53 by high glucose. Exposure to high-glucose medium increased p53 activity in mock-infected cells (Figure 2A), although this increase was significantly inhibited by introduction of SIRT1 or ablation of p53 (data not shown). These results indicated that downregulation of SIRT1 expression was responsible for high glucose-induced senescence. Moreover, the induction of ICAM-1 and p21 expression by exposure to high-glucose medium was inhibited by either introduction of SIRT1 or ablation of p53 activity (Figure 2C), suggesting a potential role of the SIRT1/p53 axis in diabetic vasculopathy.

**Decreased Expression of Sirt1 Is Associated With Vascular Senescence in Diabetic Mice**

To further investigate whether Sirt1 is involved in the pathogenesis of diabetic vasculopathy, we produced a mouse model of diabetes by treatment with streptozotocin and harvested the aorta after 4 weeks. Western blot analysis revealed that expression level of Sirt1 was significantly lower in the aortas of diabetic mice than in those of nondiabetic mice (Figure 3A and supplemental Figure IIA). Consistent with this finding, levels of acetylated p53 and p21 expression were significantly higher in the aortas of diabetic mice compared with those of control mice (Figure 3A and 3B and supplemental Figure IIB). Histological analyses revealed that an increase of senescence-associated β galactosidase activity (a biomarker for cellular senescence) was predominantly observed in aortic endothelial cells of diabetic mice and that this increase was associated with downregulation of Sirt1 expression and upregulation of p53 expression in aortic endothelial cells (Figure 3C and supplemental Figure IIB). These results suggest that hyperglycemia induced p53-dependent endothelial cell senescence. Furthermore, the expression of ICAM-1 was significantly increased in aortic endothelial cells of diabetic mice (Figure 3A and supplemental Figure IIA and IIB). We thus speculated that activation of Sirt1 might improve vascular dysfunction in diabetic mice.

**Resveratrol Improves Vascular Dysfunction in Diabetic Mice**

To test our hypothesis, we treated diabetic mice with resveratrol, a sirtuin activator. Injection of streptozotocin markedly decreased the plasma insulin below detectable levels and
the plasma glucose level gradually increased (supplemental Figure IIIA). Resveratrol did not affect the increase of plasma glucose after streptozotocin treatment (supplemental Figure IIIA). Indeed, there were no differences of plasma insulin, cholesteral, or triglyceride levels between vehicle-treated and resveratrol-treated diabetic mice (supplemental Figure IIIA and data not shown). Thus, resveratrol did not seem to improve the diabetic state in this experimental setting. However, resveratrol reduced the acetylated p53 level and suppressed induction of p21 expression in the aortas of diabetic mice (Figure 4A and 4B and supplemental Figure IIB and IIIC), suggesting that activation of sirtuins prevented vascular cell senescence accelerated by hyperglycemia. An increase of leukocyte rolling and adhesion is known to be an initial step in the development of atherosclerosis, and ICAM-1 is thought to be a key endothelial receptor involved in these events. Therefore, we examined the effects of resveratrol on ICAM-1 expression in the aortas of diabetic mice. We found that the expression of ICAM-1 was significantly lower in resveratrol-treated diabetic mice compared with vehicle-treated mice (Figure 4A and supplemental Figure IIIB). Next, we investigated whether resveratrol reduced leukocyte rolling and adhesion in diabetic mice. Using an intravitral microscopy, we observed that hyperglycemia significantly promoted leukocyte rolling and adhesion in the femoral artery, whereas these changes were markedly inhibited by treatment with resveratrol as well as by administration of anti–ICAM-1 neutralizing antibody (Figure 4C and supplemental movies), indicating that resveratrol treatment downregulates ICAM-1 expression, thereby inhibiting leukocyte rolling and adhesion. In contrast, resveratrol had little influence on aortic ICAM-1 expression or leukocyte rolling in p53-deficient mice (data not shown), suggesting that resveratrol inhibited p53-dependent vascular cell senescence induced by hyperglycemia and thereby protected diabetic mice against vascular dysfunction. Protective roles of Sirt1 in diabetic vasculopathy were further supported by the observations that resveratrol treatment significantly increased neovascularization in ischemic limbs and nitric oxide synthase activity in diabetic mice (Figure 4D and 4E). Thus, activation of Sirt1 may be a novel strategy for the treatment of diabetic vascular complications.

The Akt/FOXO Pathway Plays a Crucial Role in the Downregulation of SIRT1 Expression by High-Glucose Conditions

The forkhead box O transcription factor (FOXO) has been shown to positively regulate SIRT1 expression.27 Because hyperglycemia has been reported to increase Akt activity,28,29 we investigated whether these signaling molecules were involved in the regulation of SIRT1 expression under high-glucose conditions. Exposure of human endothelial cells to a high concentration of glucose led to an increase of phospho-Akt (supplemental Figure IVA). When this pathway was disrupted by introducing a dominant-negative form of Akt, exposure of cells to high glucose failed to affect the levels of SIRT1 and acetylated p53 (supplemental Figure IVB), suggesting a critical role of the Akt signaling pathway in the downregulation of SIRT1 expression by high-glucose conditions.

Discussion

In the present study, we demonstrated a novel mechanism of diabetic vasculopathy. Hyperglycemia reduces Sirt1 expression, leading to p53-dependent vascular cell senescence and thus to vascular dysfunction. It remains unclear how hyperglycemia downregulates Sirt1 expression. FOXO has been shown to positively regulate SIRT1 expression.27 Akt phosphorylates FOXO and thus blocks its transcriptional activity by promoting cytoplasmic retention and degradation, and it has been reported that hyperglycemia upregulates Akt activity.28,29 The AMP-activated protein kinase (AMPK) plays a critical role in the cellular responses to low energy levels, and phosphorylation by AMPK leads to the activation of FOXO transcriptional activity without affecting FOXO subcellular localization.30 Because phospho-Akt levels were increased and phospho-AMPK levels were reduced in the aortas of diabetic mice compared with those of nondiabetic mice (unpublished data, 2008), hyperglycemia may downregulate expression of Sirt1 by decreasing FOXO activity. Consistent with...
this idea, our in vitro experiments showed that treatment of human endothelial cells with high glucose led to activation of Akt and a decrease of SIRT1 expression. This decrease was significantly inhibited by introduction of a dominant-negative form of Akt, suggesting that the downregulation of SIRT1 expression by high-glucose conditions is at least partially mediated by the Akt/FOXO signaling pathway.

SIRT1-mediated deacetylation of p53 prevents p53-dependent transactivation of various target genes such as p21 and Bax. These direct effects of SIRT1 on p53 transactivation are important for the function of p53 as a transcription factor because the acetylation status has been shown to be indispensable for its ability to repress cell growth and induce apoptosis.31 It has been reported that the inhibition of cellular deacetylases leads to a longer half-life for endogenous p53, indicating that acetylation of p53 also contributes to p53 stabilization.32 Thus, SIRT1 may negatively regulate the ability of p53 to promote endothelial senescence by inhibiting its transcriptional activity as well as by inducing its degradation. In addition to being a direct effector of SIRT1 deacetylation, p53 can repress SIRT1 transcription by binding to 2 response elements within the SIRT1 promoter,27 which suggests that SIRT1 and p53 exist in a negative-regulatory feedback loop: hyperglycemia-induced downregulation of SIRT1 may further decrease its expression via p53 activation.

We have previously reported that activation of the insulin/Akt pathway enhances the aging of cultured human endothelial cells via the p53/p21-dependent pathway.33 This effect is partly mediated by a decrease of FOXO activity, which leads to downregulation of antioxidant genes and an increase of the intracellular level of reactive oxygen species (ROS).34 Therefore, both hyperinsulinemia and hyperglycemia may induce vascular cell senescence through mechanisms involving SIRT1-dependent and -independent pathways, thereby promoting vascular complications in patients with type 2 diabetes. An increase of oxidative stress is associated with most of the pathways that have been implicated in diabetic vasculopathy including the polyol pathway and the protein kinase C pathway.35 Thus, these pathways may also promote p53-dependent vascular cell senescence by increasing ROS levels.

In addition to p21 expression, ICAM-1 was induced by hyperglycemia, and its induction was disrupted by ablation of p53. This finding was in accordance with a previous report that p53 directly activates ICAM-1 expression in an NF-κB-independent manner.36 Senescent cells also exhibit various features of endothelial dysfunction such as decreased production of nitric oxide and increased expression of cytokines and coagulants.37 Thus, inhibition of vascular cell senescence by activation of SIRT1 may be a potential therapeutic strategy for human vascular diseases.

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Disclosures

None.

References


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Supplemental Materials and Methods

Mouse study
All animal study protocols were approved by the Chiba University review board. C57/BL6 mice were purchased from the SLC Japan. p53-deficient mice (C57/BL6 background) were purchased from the Jackson Laboratory. For the diabetic model, mice were treated with an intraperitoneal injection of streptozotocin in 0.1 M sodium citrate (pH 4.5) at a dose of 50 mg/kg body weight for 5 days. Leukocyte rolling assay was performed as described previously with modifications\(^1\). Briefly, 8-week-old mice were anesthetized by injection of pentobarbital sodium (50 mg/kg body weight ip). The left jugular vein was cannulated to administer rhodamine 6G dye and additional anesthetic agents. An incision was made in the thigh skin to expose the femoral artery. The exposed tissue was superfused with 37\(^\circ\)C warmed phosphate-buffered saline, pH 7.4. An intravital microscope (Axiolinput; Carl Zeiss MicroImaging, Inc.) with a \(\times25\) objective lens (Weltzlar L25/0.35; E. Leitz Inc.), and a \(\times10\) eyepiece was used to examine leukocyte rolling. A high-resolution black-and-white camera was used to project the images onto a monitor, and the images were recorded (30 frames/second for 1 minute) for analysis using a personal computer. The number of rolling leukocytes was counted in each frame, and the cumulative number was calculated. Intraperitoneal injection of anti-ICAM-1 antibody (3 mg/kg, BD Bioscience) was performed at 3 days before the assay. Estimation of neovascularization in a hind limb ischemia model and measurement of NOS activity were performed as described previously\(^2,3\).
Supplemental figure legends

**Figure I  Relative expression of SIRT1, p53, p21 and ICAM-1.**

Human endothelial cells were cultured in medium containing various concentrations of glucose (0, 100, 400 mg/dl) for 24 hours and harvested to examine SIRT1, acetylated p53 (Ac-p53), p53, p21, and ICAM-1 levels by Western blot analysis.  *P<0.05 versus 0 mg/dl glucose; #P<0.05 versus 100 mg/dl glucose.  Error bars indicate s.e.m.; n = 3.

**Figure II  Expression of Sirt1, p53, p21, Icam-1, pAkt, and Foxo in STZ-treated mice.**

(A) Mice were treated with vehicle (−) or streptozotocin (STZ) (+). The aortas were harvested 4 weeks after treatment, and Western blot and Northern blot analyses were performed.  *P<0.05 versus STZ (−). Error bars indicate s.e.m.; n = 4–6.  (B) Immunohistochemistry for Sirt1, p53, and Icam-1 (brown) in the aortas. Down-regulation of Sirt1 expression and up-regulation of p53 expression were observed in aortic endothelial cells of diabetic mice.  Scale bar: 10 μm.

**Figure III  Effects of resveratrol on insulin and glucose levels and expression of acetyl p53, p21, and Icam-1.**

(A–C) Mice were given vehicle (−) or streptozotocin (+) and treated with resveratrol (Res) or vehicle. Plasma insulin and glucose levels were measured (A). Resveratrol did not seem to improve the diabetic state in this experimental setting.  *P<0.05 versus STZ (−)/Vehicle; #P<0.05 versus STZ (−)/Res.  Error bars indicate s.e.m.; n = 3–5. The aortas from the mice were analyzed for Acetylated p53 (Ac-p53), Icam-1 (B), and p21 expression (C).  *P<0.05 versus STZ (−)/Vehicle; #P<0.05 versus STZ (+)/Vehicle.  Error bars indicate s.e.m.; n = 3–4.
Figure IV  Effects of high glucose conditions on levels of phospho-Akt, SIRT1, and acetylated p53.

(A) Human endothelial cells were exposed to high glucose (400 mg/dl) and harvested to examine for phospho-Akt (pAkt) and Akt levels by Western blot analysis.  (B) Human endothelial cells were infected with pLNCX (Mock) or pLNCX DN-Akt (DN-Akt), after which cells were exposed to glucose at indicated concentrations. SIRT1 and acetylated p53 levels were examined by Western blot analysis.  *P<0.05 versus 0 mg/dl glucose/Mock.  Error bars indicate s.e.m.; n = 3.

Supplemental movies legends

Leukocyte rolling assay.
Mice were given vehicle (−) or streptozotocin (+) and treated with resveratrol (Res) or vehicle. Leukocyte rolling assay was performed 4 weeks after treatment.  A, Vehicle-treated non-diabetic mice.  B, Vehicle-treated diabetic mice.  C, Resveratrol-treated diabetic mice.
Supplemental References


Supplementary Fig I

**SIRT1**

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