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Dapagliflozin impedes endothelial cell senescence by activating the SIRT1 signaling pathway in type 2 diabetes

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ABSTRACT

Background: Sodium-glucose cotransporter 2 inhibitors (SGLT2i) clinically reduce atherosclerosis and lower blood pressure. However, their impact on endothelial dysfunction in type 2 diabetes (T2D) remains unclear. In this study, we investigated the protective effect and underlying mechanism of the SGLT2 inhibitor dapagliflozin in diabetes.

Methods: Vascular reactivity was measured to assess the vasoprotective effect of dapagliflozin in a mouse model of high glucose (HG)-induced T2D. Pulse wave velocity was measured to quantify arterial stiffness. Protein expression was assessed by western blotting and immunofluorescence, oxidative stress was evaluated using dihydroethidium, nitric oxide was evaluated using the Griess reaction, and cellular senescence was assessed based on senescence-associated beta-galactosidase (SA-β-gal) activity and the expression of senescence markers. Furthermore, the endothelial nitric oxide synthase (eNOS) acetylation status was determined and eNOS interactions with SIRT1 were evaluated by coimmunoprecipitation assays.

Results: Dapagliflozin protected against impaired endothelium-dependent vasorelaxation and improved arterial stiffness in the mouse model of T2D; mouse aortas had significantly reduced levels of senescence activity and senescence-associated inflammatory factors. HG-induced increases in senescence activity, protein marker levels, and oxidative stress in vitro were all ameliorated by dapagliflozin. The decreases in eNOS phosphorylation and nitric oxide (NO) production in senescent endothelial cells were restored by dapagliflozin. SIRT1 expression was reduced in HG-induced senescent endothelial cells, and dapagliflozin restored SIRT1 expression. SIRT1 inhibition diminished the antisenescence effects of dapagliflozin. Coimmunoprecipitation showed that SIRT1 was physically associated with eNOS, suggesting that the effects of dapagliflozin are dependent on SIRT1 activation.

Conclusion: These findings indicate that dapagliflozin protects against endothelial cell senescence by regulating SIRT1 signaling in diabetic mice.

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1. Introduction

Type 2 diabetes (T2D) has serious consequences, particularly macrovascular complications (including ischemic heart and cerebrovascular disease) and microvascular complications (including nephropathy and neuropathy), which increase the risk of premature death from cardiovascular disease. T2D represents one of the greatest global chronic disease pandemics to date [1–4].

Endothelial cell senescence contributes to the premature reduction of vascular function in patients with diabetes [5]. It is associated with structural and functional alterations, changes in gene expression, increased oxidative stress, and decreased endothelial nitric oxide synthase (eNOS)-based production of nitric oxide (NO), increasing pro-atherothrombotic responses [6,7]. Senescent endothelial cells have been observed in the aortas of diabetic rats [6–8] after exposure to a high glucose (HG) concentration, with an increased abundance of senescence-associated β-galactosidase (SA-β-gal)-positive cells [6,7]. The development of novel therapies for delaying endothelial cell senescence is thus an important aim for preventing diabetic vascular complications.

Recent clinical studies have showed the favorable effects of sodium-glucose cotransporter 2 inhibitors (SGLT2i) on the risk of atherosclerotic cardiovascular mortality in patients with T2D [9–11]. These findings have revolutionized the care of patients with T2D. Further work has demonstrated that SGLT2i lowers systolic and diastolic pressures in patients without diabetes [12]. Consequently, numerous large-scale clinical studies have investigated endothelium-dependent vascular function in T2D treated with SGLT2i. The DEFENCE study demonstrated a considerable improvement in vascular endothelial function in T2D receiving dapagliflozin, an SGLT2i, in conjunction with metformin [13]. Another single-center, randomized clinical trial revealed that dapagliflozin improved endothelial dysfunction in T2D [14]. Solini et al. reported that dapagliflozin has a cardioprotective effect by preserving the vasodilatory capacity [15]. Animal studies have revealed that dapagliflozin improves arterial stiffness in T2D mouse models [16,17]. However, the mechanism by which dapagliflozin exerts vasoprotective effects remains unclear and was an objective of the current study.

The ability of SGLT2 to promote paradigm shift and induce a state of fasting mimicry may explain its cardiovascular protective effects [18]. In particular, the SGLT2i-induced activation of the sirtuin 1 (SIRT1)/AMPK pathway and suppression of Akt/mTOR signaling led to a reduction in oxidative stress and suppression of inflammation [19]. In the vasculature, SIRT1 is expressed in endothelial cells and synergistically regulated by eNOS to maintain cell senescence [20]. These observations demonstrate the pivotal role of the SIRT1 pathway in diabetic vascular complications. This study aimed to evaluate whether dapagliflozin prevents endothelial cell senescence and to investigate whether SIRT1 signaling is associated with the protective effects of dapagliflozin. These findings improve our current understanding of how dapagliflozin prevents atherosclerotic events in T2D and provide evidence for its therapeutic effects against atherosclerotic cardiovascular disease.

2. Materials and methods

2.1. Animals

Eight-week-old male mice possessing a homozygous point mutation in the leptin receptor gene (C57BLKS/J-lepr^{db}/lepr^{db}: db/db) and age- and gender-matched heterozygous littermates (C57BLKS/J-lepr^{db}/+: db/m) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The db/db mouse model was suitable model as it develops T2D by 6–8 weeks of age, as shown by the presence of obesity, hyperglycemia, insulin resistance, and dyslipidemia. Mice were provided *ad libitum* access to food and water and were housed at a temperature of 22 ± 2 °C under a 12-h/12-h light/dark cycle.

2.2. Dapagliflozin treatment

After acclimatization, mice were randomized into db/m + saline, db/m + dapagliflozin, db/db + saline, and db/db + dapagliflozin groups (n = 15). As described in a previous study [16], chronic dapagliflozin (MedChemExpress, Monmouth Junction, NJ, USA, #BMS-512148) administration via gavage (8 weeks, 1.0 mg/kg/day) was used to investigate vascular protection in diabetic mice. Placebo groups were given a similar daily oral dose of vehicle (saline).

2.3. Glucose and lipid metabolism measurements

Blood glucose levels were measured weekly after 6 h of fasting with an Accu-Chek Aviva glucose analyzer (Roche Diagnostics, Rotkreuz, Switzerland). A subset of mice (n = 7–8 per group) was subjected to intraperitoneal glucose tolerance tests (IGTTs) after a 6-week intervention period. After 4 h of fasting, mice were injected with a bolus of glucose (1 mg/g body weight) and tail-vein blood was sampled at 0, 15, 30, 60, and 120 min after glucose injection.

Blood samples collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant were used to quantitatively evaluate glucose and lipid metabolism. Samples were centrifuged for 15 min at 1000×*g* at 2–8 °C within 30 min of collection. Insulin and triglyceride levels were measured using an ultrasensitive mouse insulin ELISA kit (CUSABIO, Wuhan, China) and a triglyceride assay kit (Sigma-Aldrich, St. Louis, MO, USA), respectively. Homeostasis model assessment for insulin resistance (HOMA-IR) was used to measure the status of insulin resistance using the formula: HOMA-IR = fasting blood glucose (mmol/L) × fasting insulin (μ/mL)/22.5.

2.4. Aortic PWV and vascular reactivity

Noninvasive aortic PWV measurement in mice was conducted as our previous study [21]. The effect of dapagliflozin on vascular reactivity was measured as described previously [16]. Briefly, aortic rings (~5 mm/ring) were mounted horizontally in a 5 mL myograph chamber (Danish Myo Technology A/S, Skejbyparken, Denmark). They were maintained at 37 °C in Krebs solution containing a 95% O_2/5% CO_2 gas mixture. Arteries were constricted using PE (10^{-6} M), followed by a dose-response analysis using endothelium-dependent dilator acetylcholine (10^{-9}–10^{-4} M). Then, a dose-response analysis using the endothelium-independent dilator sodium nitroprusside (SNP; 10^{-9}-10^{-4} M) was conducted following pre-constriction with PE (10^{-6} M).

2.5. Inflammatory cytokines

Plasma samples were analyzed in duplicate on microspheres to measure the concentrations of circulating inflammatory markers: interleukins (IL)-8, −17A, and −17F by fluorescent microbead-based flow cytometry (Luminex multiplex flow cytometer, Thermo Fisher, Waltham, MA, USA). The intra-assay variability (<5%) was within the normal limits reported by the manufacturer.

2.6. Chemiluminescent detection of NO

To measure eNOS activity in endothelial cells, NO production was measured using the Griess reaction. Cell culture supernatants or plasma samples were mixed with equal volumes of nitrate/nitrites and the nitrite concentration was measured using a Nitrate/Nitrite Assay Kit (Sigma, St. Louis, MO, USA) following the manufacturer's protocol.

2.7. Immunofluorescence, histological analysis, and immunohistochemistry

To detect senescence markers in murine aorta sections, after antigen retrieval tissue sections were incubated overnight (12 h) at 4 °C with antibodies against p21 (1:600; Proteintech, Rosemont, IL, USA, #10355–1-AP), p53 (1:100; Proteintech, #10442–1-AP), and CD31 (1:50; Santa Cruz Biotechnologies, Dallas, TX, USA, #sc-376764). Sections were then incubated at 37 °C for 1 h with goat anti-mouse or anti-rabbit fluorescein isothiocyanate- or tetramethyl-rhodamine isothiocyanate-linked secondary antibodies (1:100). Finally, the sections were counterstained with DAPI. Nonspecific IgG-stained sections were used as negative controls. Images were acquired using an Olympus microscope (Tokyo, Japan; × 200 magnification). Color composite images were generated and analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA). Histological analysis and immunohistochemistry were conducted as our previous study [21].

2.8. Cell culture

HUVECs were (Carlsbad, CA, USA #8000) cultured at 37 °C in a humidified atmosphere and 5% CO_2. The medium was comprised of a base medium supplemented with 5% fetal bovine serum, 1% endothelial cell growth supplement (ScienCell, #1001), 100 units/mL penicillin, and 100 μg/mL streptomycin. HUVEC monolayers (passage 4) were harvested at sub-confluence, seeded into 10 cm dishes, and exposed to experimental conditions for 6 days. According to previous studies [22], 10 μM dapagliflozin was administered to HUVECs under the following conditions: (1) normal glucose medium (5.5 mM), (2) normal glucose medium plus 10 μM dapagliflozin, (3) HG medium (30 mM), (4) HG medium plus 10 μM dapagliflozin, (5) mannitol medium (30 mM), and (6) mannitol medium plus 10 μM dapagliflozin. Mannitol was used to reduce osmotic effects.

2.9. SA-β-gal staining

HG-induced senescent HUVECs pretreated with dapagliflozin (10 μM) for 2 h were grown in HG (30 mM) medium for 6 days, with a medium change and reintervention every 24 h, followed by incubation with SA-β-gal labeling reagent (Beyotime, Shanghai, China; C0602). After wash, cells were fixed for 15 min using fixation buffer (Beyotime, C0602) and incubated for 12 h at 37 °C with SA-β-gal staining solution. SA-β-gal staining was also performed to assess the detailed effects of dapagliflozin on tunica intima senescence as described previously [23].

2.10. NAD^+/NADH ratios and ROS quantification

To measure SIRT1 activity, NADt (total NAD^+ + NADH) was extracted from HUVECs following the manufacturer's instructions and NAD^+/NADH ratios were measured using an NAD^+/NADH Quantification Kit (Beyotime, #S0175). To determine the effect of dapagliflozin on senescence-associated oxidative stress in HUVECs, the fluorescent probe dihydroethidium (DHE) was used to measure intracellular reactive oxygen species (ROS) production [24].

2.11. Western blotting and protein acetylation

HUVECs were homogenized in RIPA lysis buffer (CWBIO, Jiangsu, China, #CW2333) containing a proteinase and phosphatase inhibitor cocktail. Equal protein quantities were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then

electro-transferred onto polyvinylidene fluoride membranes. The blots were blocked in 5% fat-free milk and probed with primary antibodies against p-eNOS, eNOS, and SIRT1 (1:1000; Cell Signaling Technologies, Inc., Danvers, MA, USA, #9570, #32027, and #9475) and p53, p21, p16, and β-actin (1:5000; Proteintech, #10442–1-AP, #10355–1-AP; and 1:1000; Proteintech, #16717-1-AP). Blots were incubated overnight (12 h) at 4 °C with primary antibodies, blocked with 5% bovine serum albumin (for phosphorylation-specific antibodies) or 5% skim milk (for other antibodies) at room temperature (20–25 °C) for 2 h, and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000; Proteintech). Antibody labeling was visualized using an electro-chemiluminescence system (Bio-Rad, Hercules, CA, USA). β-Actin was used as loading controls. Immunoprecipitation was applied to isolate eNOS and quantify its acetylation as described previously [24]. Anti-SIRT1 (1:1000; Cell Signaling Technologies, Inc., #9475) and anti-acetylated lysine antibodies (1:1000; Cell Signaling Technologies, Inc., #9441) were used.

2.12. Statistical analyses

Data were collected from at least three independent experiments and are presented as means ± standard deviation. All data were analyzed using GraphPad Prism v8.0 (GraphPad Inc., La Jolla, CA, USA). For normally distributed data, pairwise differences between groups were analyzed using *t*-tests, and differences among multiple groups were analyzed using a one-way analysis of variance,

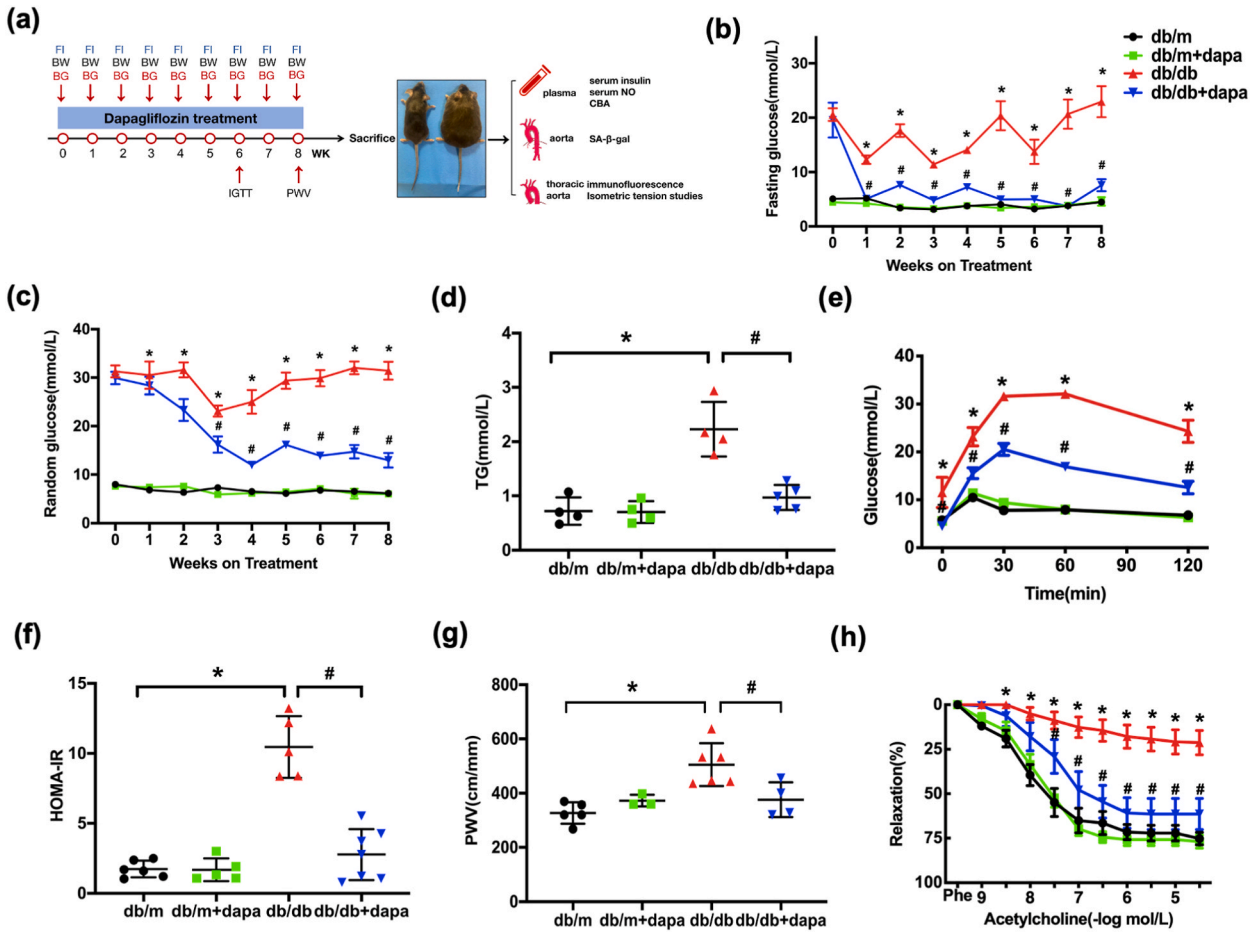

Fig. 1. Dapagliflozin protects against endothelium-dependent vasorelaxation in murine type 2 diabetes (T2D). (a) Strategy for dapagliflozin treatment and measurement of metabolic parameters (left). Scheme for blood collection, tissue extraction, and organ morphometry (right). Eight-week-old control and diabetic mice were randomly divided into four groups: (1) control receiving saline (db/m + saline, n = 17), (2) control receiving dapagliflozin (db/m + dapa, n = 14), (3) diabetic receiving saline (db/db + saline, n = 13), (4) diabetic receiving dapagliflozin (db/db + dapa, n = 15). **(b, c, and d)** Glucose and lipid metabolic measurements showing that T2D db/db mice had significantly higher fasting and non-fasting blood glucose and total triglyceride levels than those of heterozygous control mice. Treatment with dapagliflozin (8 weeks, 1.0 mg/kg/day) reversed these metabolic effects. (**e and f)** Impaired glucose tolerance test (IGTT) and homeostasis model assessment for insulin resistance (HOMA-IR) showing that T2D db/db mice had impaired glucose handling and significant insulin resistance, and these changes were attenuated by dapagliflozin treatment. (**g)** Pulse wave velocity (PWV) measurements to detect arterial stiffness. Dapagliflozin decreased PWV. (**h)** Vascular reactivity assessed to measure the vasoprotective effect of dapagliflozin. Data are presented as means ± SD. *P < 0.05 versus db/m; #P < 0.05 versus db/db. Dapa, dapagliflozin; FI, food intake; BW, body weight; BG, blood glucose.

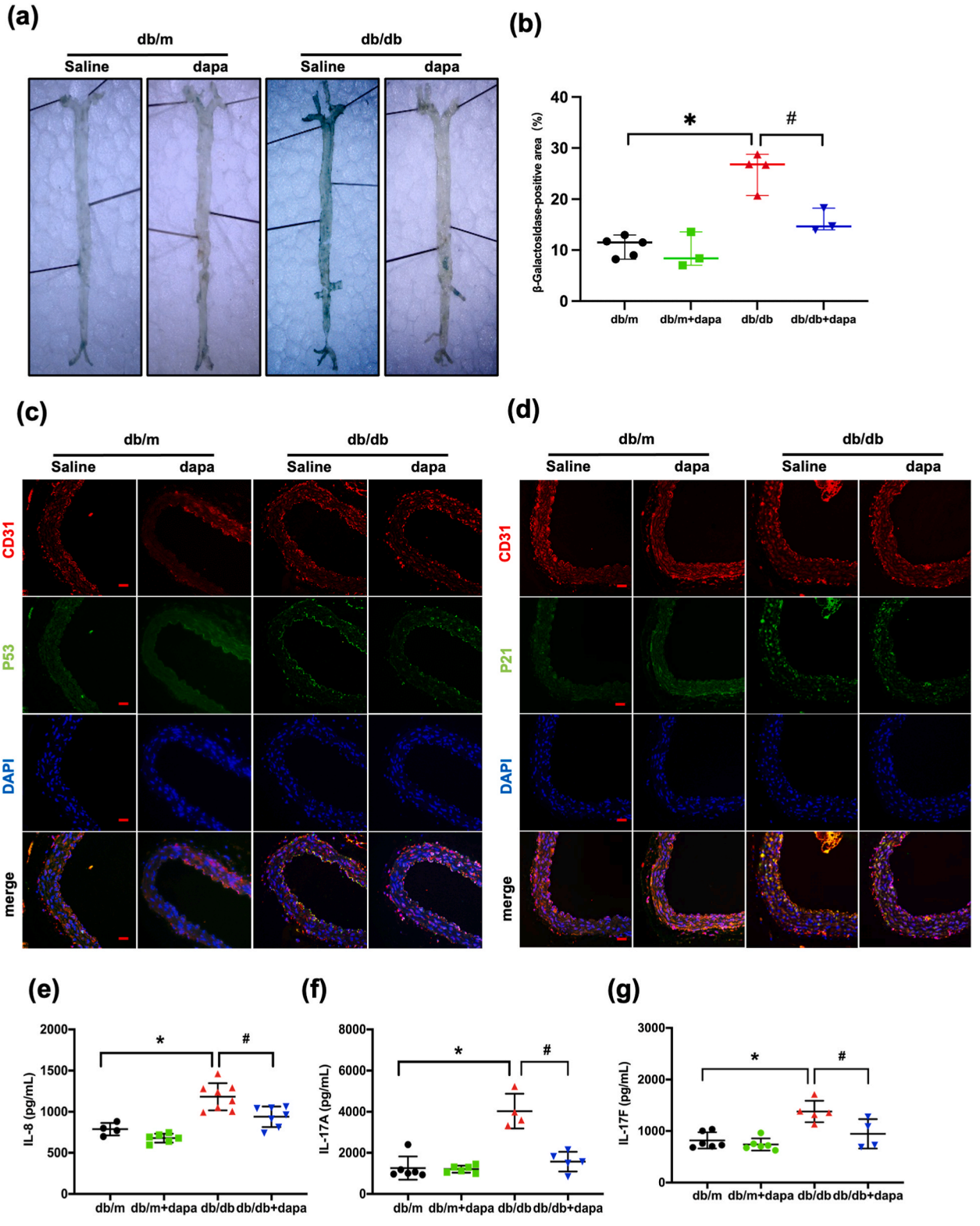

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Fig. 2. Dapagliflozin prevents endothelial cell senescence in T2D mice. (a and b) Senescence-associated β-galactosidase (SA-β-gal) staining of aortas (β-galactosidase staining is blue); n = 3–5 showing senescence. (**c and d)** Immunofluorescent detection of senescence markers in aortas. CD31 was used to identify endothelial cells and p21 and p53 protein levels were detected to assess senescence. n = 6, Scale bar = 100 μm. **(e, f, and g)** Flow-based fluorescence immune-microbead assay to measure levels of circulating inflammatory cytokines IL-8, IL-17A, and IL-17F. n = 4–8. Data are presented as means ± SD. *P < 0.05, versus db/m; #P < 0.05 versus db/db. Dapa, dapagliflozin.

followed by Tukey's post-hoc tests. For non-normally distributed data, pairwise differences were evaluated using Mann–Whitney U tests, and multiple groups were compared using Kruskal–Wallis tests. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Dapagliflozin protects against impaired endothelium-dependent vasorelaxation in T2D mice

To assess the effects of dapagliflozin on diabetic vasculature, 8-week-old male diabetic (db/db) and age-matched non-diabetic (db/m) control mice were randomized into db/m, db/m + dapagliflozin, db/db, and db/db + dapagliflozin groups (Fig. 1a).

T2D mice had significantly higher blood glucose and total triglyceride levels than those of heterozygous control mice. Treatment with dapagliflozin reversed these metabolic findings (Fig. 1b, c, and d). T2D db/db mice possessed impaired glucose handling, evidenced by higher glucose levels during the IGTT, as well as significant insulin resistance, as indicated by elevated HOMA-IR values. However, both of these parameters were improved by treatment with dapagliflozin (Fig. 1e and f).

To evaluate the effect of dapagliflozin on arterial stiffness, pulse wave velocity (PWV) as a vascular senescence marker was measured after chronic dapagliflozin administration via gavage for 8 weeks. The PWV of diabetic mice was higher than that of age-matched control mice; this difference was attenuated by dapagliflozin (Fig. 1g). A morphometric analysis was performed after HE staining. Aortas in the db/db mice significantly higher medial layer thicknesses and medial cross-sectional areas than those of heterozygous wild-type controls; moreover, these increases were alleviated by dapagliflozin; however, the differences were not statistically significant. There were no significant differences in the lumen diameter and medial layer thicknesses/lumen diameter ratio of the aortas among the three groups (Supplementary Figs. 1a–e). We evaluated changes in collagen by Masson's trichrome staining of the aortic tissue rings. The collagen content was elevated in diabetic mice, and these increases were attenuated by dapagliflozin (Supplementary Figs. 1e and f). These results show that dapagliflozin plays a key role in preventing the functional and structural alterations that occur during diabetic vascular senescence. To assess the vasorelaxation protective effect of dapagliflozin in adult diabetic mice, vascular reactivity was measured by measuring the isometric tension. There was a significant improvement in endothelium-dependent vasorelaxation in the aortas of dapagliflozin-treated diabetic mice compared to that of vehicle controls (Fig. 1h). However, endothelium-independent vasorelaxation mediated by SNP was not affected by chronic dapagliflozin treatment. These data suggest that dapagliflozin protects against endothelium-dependent vasorelaxation in the murine db/db vasculature.

3.2. Dapagliflozin prevents endothelial cell senescence

The aortas of diabetic mice demonstrated higher SA-β-gal levels than those of control mice; dapagliflozin strongly decreased senescence activity in diabetic mice (Fig. 2a and b). Immunofluorescence indicated that the senescence markers p53 and p21 were upregulated in diabetic mice; these increases in expression were attenuated by dapagliflozin (Fig. 2c and d). Additionally, we found that the secretion of circulating inflammatory factors, including IL-8, IL-17A, and IL-17F, was significantly elevated in db/db mice and these increases were reversed by dapagliflozin (Fig. 2e, f, and g). Collectively, these data demonstrate that dapagliflozin prevents vascular senescence in T2D mice.

The effects of dapagliflozin on senescence in cultured HUVECs were similar. The fraction of SA-β-gal positive cells increased after HG treatment (Fig. 3a and b), accompanied by increases in p53, p21, and p16 expression (Fig. 3c, d, and e; Supplementary Fig. 2). Interestingly, dapagliflozin reversed the HG-induced increases in senescence cells as well as p53, p21, and p16 expression (Fig. 3b, d, and e; Supplementary Fig. 2). By contrast, no such effect was observed after treatment with mannitol, ruling out osmotic effects. These results suggest that dapagliflozin mitigates premature senescence in endothelial cells.

3.3. Dapagliflozin restores eNOS activity and mitigates oxidative stress in senescent endothelial cells

Endothelium-derived NO is important for normal vascular function. To detect whether NO production and eNOS activity are diminished in senescent endothelial cells, we measured NO production using the Griess reaction. Plasma levels of NO decreased in db/db mice, and this decrease was reversed by dapagliflozin (Fig. 3f). Oxidative stress is a hallmark of endothelial cell senescence. DHE was used to detect ROS in HUVECs for analyzing the effect of dapagliflozin on oxidative stress. Representative images show that dapagliflozin inhibited the ROS in HUVECs (Fig. 3g and h). These results indicate that dapagliflozin restores NO production and mitigates oxidative stress in senescent HUVECs.

eNOS, as an important source of NO, is dynamically regulated by protein phosphorylation. We found that total eNOS expression was not significantly affected by dapagliflozin treatment in HG-induced senescent endothelial cells. However, phosphorylation at eNOS Ser-1177 was reduced in HG-induced senescent endothelial cells, and dapagliflozin reversed this effect (Fig. 3i and j). These findings suggest that dapagliflozin exerts its effects on eNOS activity by regulating eNOS phosphorylation in premature senescent

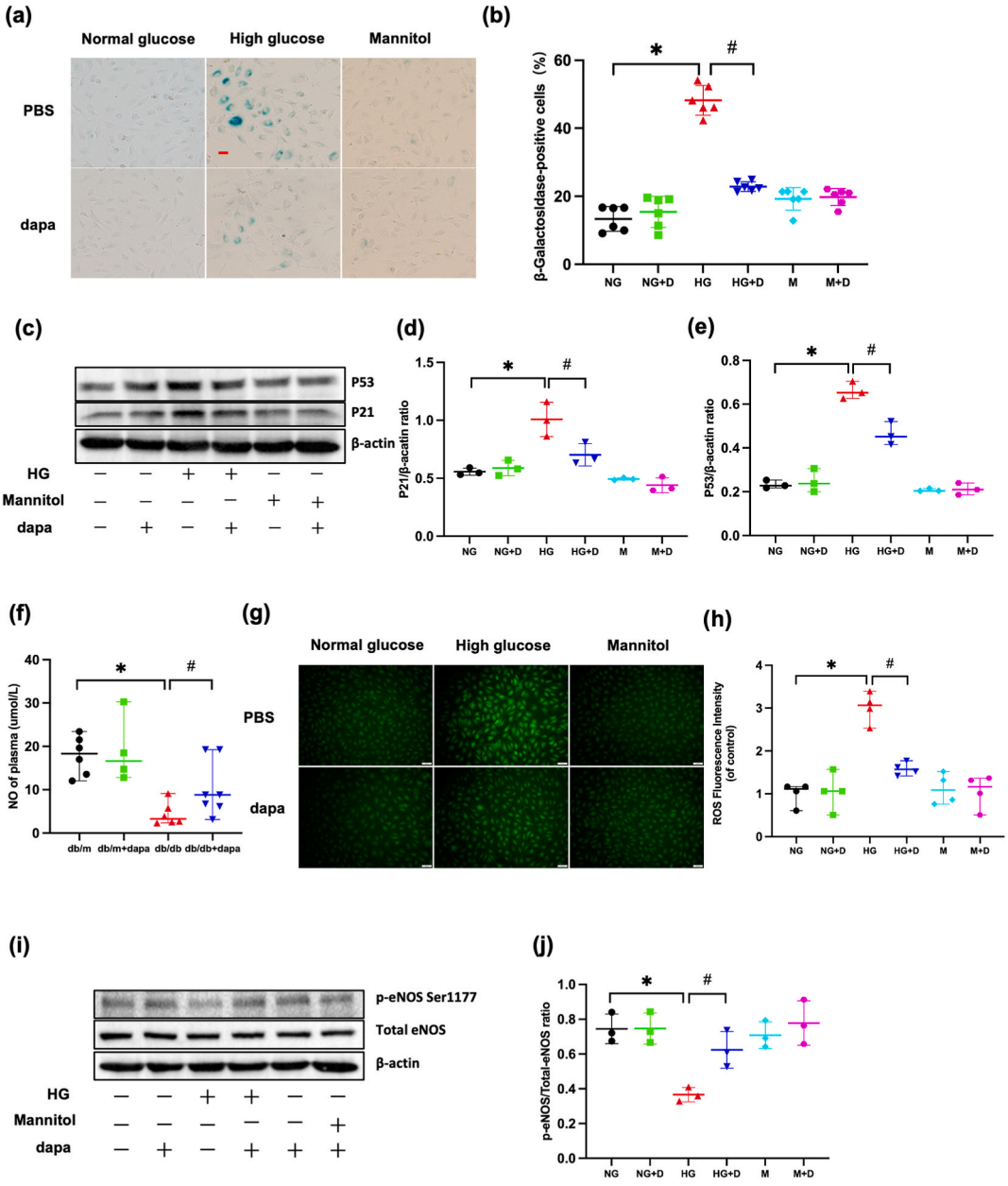

Fig. 3. Dapagliflozin restores endothelial nitric oxide synthase (eNOS) activity and mitigates oxidative stress in senescent endothelial cells. (a and b) Senescence-associated β-galactosidase (SA-β-gal) staining to detect senescence in endothelial cells stimulated with HG (30 mM). Mannitol was used as a control to rule out osmotic effects. n = 6–8, scale bar = 50 μm. Data are presented as means ± SD. *P < 0.05 versus normal glucose; #P < 0.05 versus high glucose. **(c, d, and e)** Immunoblotting to measure p53 and p21 protein levels in HG-stimulated endothelial cells. β-Actin was used for normalization. n = 3. Data are presented as means ± SD. *P < 0.05, versus normal glucose; #P < 0.05 versus high glucose. **(f)** Griess reaction to detect endothelial nitric oxide (NO) production in the plasma of db/db mice. NO levels decreased in the plasma of db/db mice, and this effect was reversed by the addition of dapagliflozin. n = 3. Data are presented as means ± SD. *P < 0.05, versus db/m; #P < 0.05 versus db/db. **(g and h)** The fluorescent probe dihydroethidium (DHE) used to detect intracellular reactive oxygen species production. n = 3, scale bar = 50 μm. **(i and j)** Immunoblotting to measure total eNOS expression and eNOS phosphorylation status. n = 3. Data are presented as mean s± SD. *P < 0.05 versus normal glucose; #P < 0.05 versus high glucose. NG, normal glucose; D, dapagliflozin; HG: high glucose; M, mannitol.

endothelial cells.

3.4. Dapagliflozin rescues SIRT1 expression in senescent endothelial cells

Clinical evidence supports the proposed conceptual framework that SGLT2i exerts its effects by activating low-energy sensors that

mimic a fasting transcriptional paradigm. We, therefore, examined the effects of dapagliflozin on the expression of SIRT1, a low-energy sensor, to assess the mechanism underlying its effect on endothelial cell senescence. Western blotting revealed that SIRT1 expression was lower in HG-induced senescent endothelial cells than in control cells and was restored by dapagliflozin (Fig. 4a and b). SIRT1 has been shown to respond to changes in nicotinamide adenine dinucleotide (NAD) levels and serves as a redox rheostat. NAD^+/NADH ratios were lower in HG-induced senescent cells than in control cells. Treatment with dapagliflozin restored the NAD^+/NADH ratio in HG-induced senescent endothelial cells (Fig. 4c). However, similar changes in the NAD^+/NADH ratio were not observed when mannitol was used for treatment, ruling out osmotic effects. To verify the role of dapagliflozin in reducing oxidative stress in the diabetic endothelium, the scavenger system, including SOD and Nrf2, was assessed. Immunohistochemical staining of the aortas revealed that SOD and Nrf2 levels were not significantly changed by dapagliflozin treatment in diabetic mice (Supplementary Figs. 3a and b). We next quantified SOD and Nrf2 levels in senescent endothelial cells, revealing no significant differences in SOD and Nrf2 expression between senescent endothelial cells with and without dapagliflozin treatment (Supplementary Figs. 3c and d). These data demonstrate SIRT1 is a pivotal downstream effector of SGLT2i in cardiovascular protection.

3.5. *SIRT1 is required for the antisenescence activity of dapagliflozin*

Nicotinamide, a pharmacological inhibitor of SIRT1, reduced the antisenescence effects of dapagliflozin in HG-treated senescent endothelial cells. In particular, the proportions of senescent cells (Fig. 4 d and e) and the expression levels of p53 and p21 (Fig. 4 f, g, and h) in HG-treated senescent endothelial cells treated with dapagliflozin were similar to those in cells without dapagliflozin

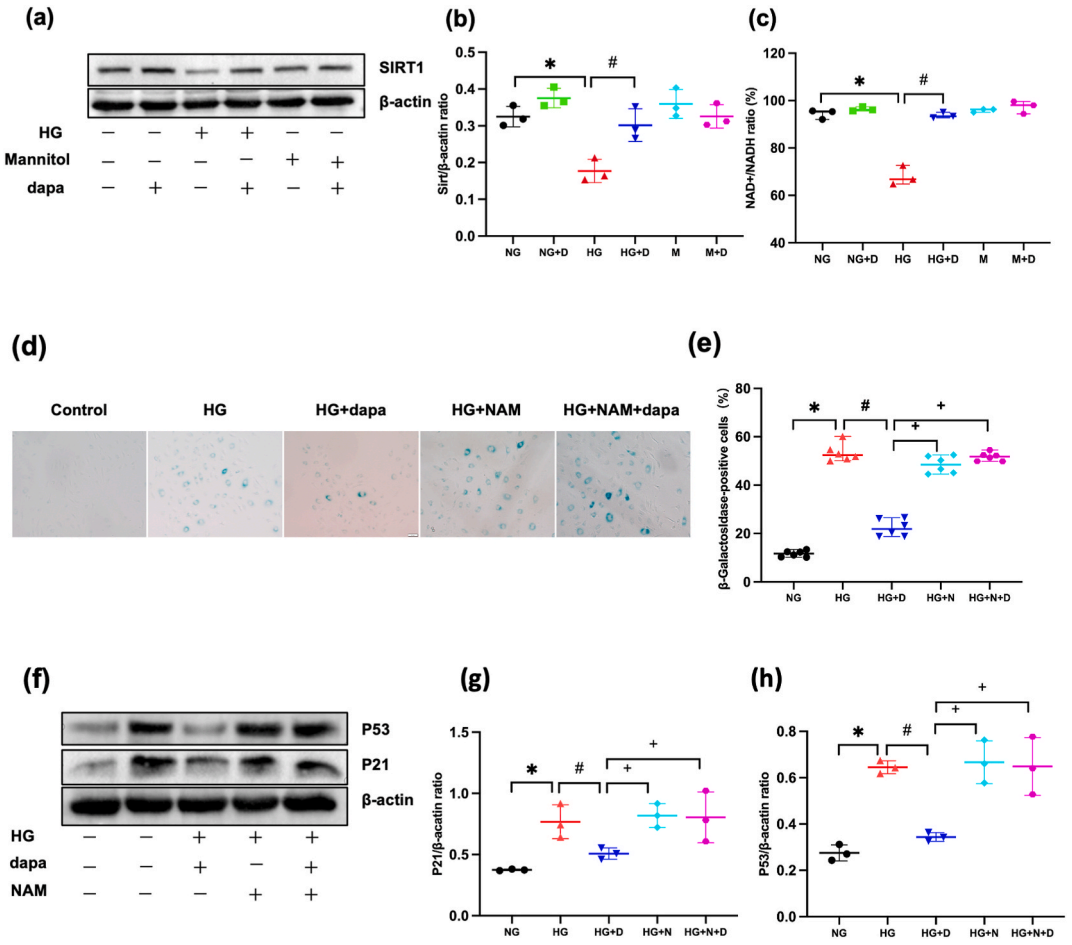

Fig. 4. Dapagliflozin rescues decreased SIRT1 expression in senescent endothelial cells. (a and b) SIRT1 expression in HG-stimulated endothelial cells normalized to β-actin levels. Mannitol was used as a control to rule out osmotic effects. n = 3. **(c)** NAD^+/NADH ratios to evaluate SIRT1 activity in HG-stimulated endothelial cells. n = 3. **(d and e)** Nicotinamide (NAM), a specific and potent pharmacological inhibitor of SIRT1, was used to inhibit SIRT1 activity. Cells were then treated with dapagliflozin and SA-β-gal staining was used to identify senescent cells. n = 6–8, Scale bar = 50 μm. **(f, g, and h)** p53 and p21 protein levels were assessed using immunoblotting, with β-actin as a loading control. n = 3. Data are presented as means ± SD. *P < 0.05, versus normal glucose; $^{\#}$P < 0.05 versus high glucose; $^{+}$P < 0.05 versus high glucose + dapa. NG, normal glucose; D, dapagliflozin; HG, high glucose; M, mannitol; N, nicotinamide.

treatment after SIRT1 inhibition. These data demonstrate that SIRT1 is required for the antisenescence effects of dapagliflozin.

SIRT1 inhibition also diminished the ROS-lowering effect of dapagliflozin in HG-treated senescent endothelial cells (Fig. 5a and b). Importantly, there were no differences in eNOS phosphorylation levels between senescent endothelial cells with and without dapagliflozin treatment after SIRT1 inhibition (Fig. 5c and d). Similar results were observed for mean aortic NAD^+/NADH ratios (Fig. 5e). These data suggest that SIRT1 is pivotal for the anti-ROS and eNOS-activating effects of dapagliflozin in HG-treated senescent endothelial cells. To further analyze the crosstalk between SIRT1 and eNOS, the acetylation status of eNOS was assessed. eNOS acetylation was higher in premature senescent endothelial cells than in control cells; this increase was attenuated by dapagliflozin. These findings indicated that SIRT1 enhances eNOS deacetylation and subsequently increases the active phosphorylation status of eNOS in premature senescent endothelial cells (Fig. 5f). Importantly, coimmunoprecipitation showed that SIRT1 was physically associated with eNOS, suggesting that SGLT2i promotes SIRT1 binding to eNOS (Fig. 5f). Taken together, these data demonstrate that dapagliflozin ameliorates vascular endothelial cell senescence by suppressing oxidative stress and promoting eNOS activation via SIRT1 signaling.

4. Discussion

The present study indicate that dapagliflozin, a selective SGLT2i, ameliorates vascular and endothelial senescence both in vivo and in vitro. Several novel findings were obtained: (1) dapagliflozin protected against impaired endothelium-dependent vasorelaxation in T2D mice, (2) dapagliflozin activated eNOS and reduced the accumulation of ROS in the diabetic vasculature and premature senescent endothelial cells, and (3) the antisenescence activity of dapagliflozin was at least partly mediated by the SIRT1 pathway. Thus, dapagliflozin provides vascular benefits by impeding diabetic endothelial cell senescence via SIRT1 activation.

Previous cardiovascular outcome trials have suggested that SGLT2i reduces the cardiovascular death in patients with T2D and atherosclerotic cardiovascular disease [9,10,25–27]. In clinical trials, dapagliflozin significantly reduced (22%) recurrent myocardial infarction in patients with T2D and prior myocardial infarction [11,28]. These findings indicate that dapagliflozin provides secondary

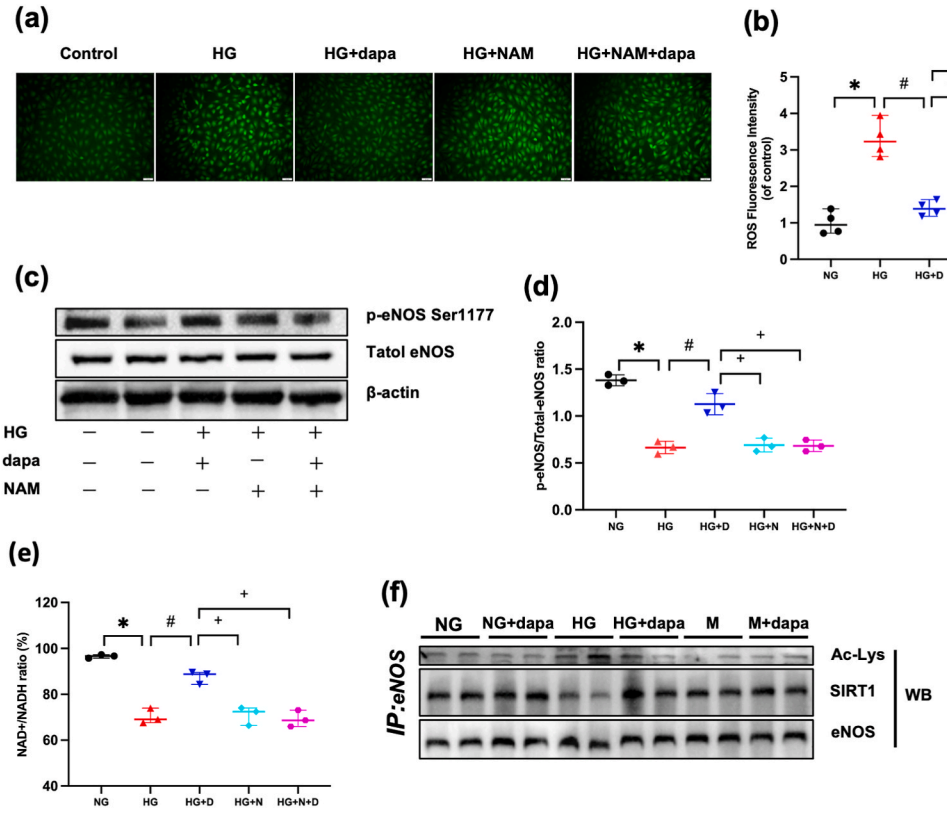

Fig. 5. SIRT1 is required for the antisenescence effects of dapagliflozin. (a and b) The fluorescence probe DHE was used to detect intracellular reactive oxygen species (ROS) production following the inhibition of SIRT1 expression. n = 3, scale bar = 50 μm. **(c and d)** Western blot analyses of total endothelial nitric oxide synthase (eNOS) expression and eNOS phosphorylation status following SIRT1 inhibition. n = 3. **(e)** NAD^+/NADH ratios determined following SIRT1 inhibition. n = 3. **(f)** Coimmunoprecipitation and immunoblotting to measure eNOS acetylation and SIRT1 levels. Data are presented as means ± SD. *P < 0.05 versus normal glucose; $^{\#}$P < 0.05 versus high glucose; $^{+}$P < 0.05 versus high glucose + dapa. NG, normal glucose; D, dapagliflozin; HG, high glucose; M, mannitol; N, nicotinamide; Ac-Lys, acetyl-lysine; ROS, reactive oxygen species; DHE, dihydroethidium.

preventive effects against atherosclerotic events in such patients [29]. Further studies should be conducted over longer time courses to assess the primary prevention of adverse cardiovascular events in patients in the absence of established atherosclerotic cardiovascular disease. Importantly, elucidating the mechanisms of action of dapagliflozin will further revolutionize the care of those patients.

The endothelium plays a crucial role in vascular homeostasis as an important barrier between the blood and the vascular walls. It is important to study the effects of dapagliflozin on the endothelium because endothelial cell senescence is an early sign of atherosclerotic cardiovascular disease. We found that dapagliflozin protects against impaired endothelium-dependent vasorelaxation, improves arterial stiffness, and prevents endothelial cell senescence in a mouse model of T2D. These findings emphasize effects of dapagliflozin on senescence and diabetic vascular complications. Previous studies have confirmed that *SGLT2* mRNA expression is undetectable in control endothelial cells but is consistently increased in endothelial cells exposed to pro-senescent stimulators (i.e., HG) [5]. These results indicate that SGLT2 is expressed in cultured and native endothelial cells under hyperglycemic conditions and dapagliflozin may exert direct effects on endothelial senescence. Recent experimental studies have revealed that SGLT2 expression promotes HG-induced cell senescence and dysfunction [30], suggesting that inhibiting SGLT2 is a potential therapeutic approach for enhancing protection against endothelial and vascular senescence.

The results of this study showed that dapagliflozin restored NAD^+/NADH ratios and SIRT1 expression in prematurely senescent endothelial cells. It has been hypothesized that SGLT2i exert cardiovascular protective effects by mimicking a state of fasting [18,31], activating SIRT1 signaling as a response to starvation and cellular stress [32]. SIRT1 plays a pivotal role as a low-energy sensor, responding to changes in NAD levels and serving as a redox rheostat [33]. Previous studies have demonstrated that SGLT2i activates SIRT1 and suppresses Akt/mTOR, consequently promoting autophagy and reducing oxidative stress independent of effects on glucose [18,19]. Importantly, dapagliflozin induced paradigm shift that resembles the cellular response to starvation, requiring SIRT1 signaling [34]. Therefore, SIRT1 may be a critical downstream component in the antisenescence and cardiovascular protective effects of dapagliflozin.

Known pathophysiological processes involved in endothelial cell senescence suggest a mechanistic association between SIRT1 and eNOS. Endothelial SIRT1 plays a vasoprotective role by regulating multiple proteins, including eNOS [35]. Reciprocal regulation between SIRT1 and eNOS has also been reported to promote endothelial cell function [36]. We found that HG significantly reduced phosphorylation at eNOS Ser-1177, whereas the treatment of HUVECs with dapagliflozin increased phosphorylation. Importantly, it has been postulated that SIRT1 increases enzymatic activity of eNOS in the endothelium by deacetylation. In the present study, acetylation of eNOS was higher in premature senescent endothelial cells, and dapagliflozin restored eNOS acetylation, indicating that SIRT1 enhances eNOS deacetylation and increases eNOS phosphorylation in premature senescent endothelial cells. Additionally, we observed that SIRT1 physically interacted with eNOS, suggesting that SGLT2i promote SIRT1 binding to eNOS. Previous studies have shown that knocking down SIRT1 or inhibiting its activity promotes eNOS acetylation; eNOS acetylation was decreased by over-expression of SIRT1 or by inducing its activity [36]. These findings indicate that SIRT1 may increase eNOS enzymatic activity via deacetylation.

Our findings show that NO production by senescent endothelial cells is reduced due to decreased eNOS phosphorylation and that treatment with dapagliflozin restores NO levels. NO is an important protective effector or signaling molecule for endothelial cell senescence [37]. There is evidence that the activation of eNOS and/or increasing NO levels in cultured cells can delay endothelial cell senescence [6,38]. In addition to eNOS activation and NO bioavailability, ROS production was strongly decreased by dapagliflozin in the present study. According to previous studies [39,40], the activation of the SIRT1 pathway downregulates ROS levels in endothelial cells, ultimately ameliorating endothelial damage. Therefore, dapagliflozin partially prevents endothelial cell senescence by increasing NO bioavailability and decreasing ROS levels via activation of the SIRT1 pathway.

This study had some limitations. First, SGLT2 knockdown/knockout studies are needed to verify the precise mechanism underlying the modulation of the SIRT1 pathway by dapagliflozin. Second, although we found that SIRT1 may mediate the antisenescence effects of dapagliflozin, further studies are warranted to determine whether the functions of targets of dapagliflozin in senescent endothelial cells are independent of SIRT1. Finally, although our findings demonstrated the antisenescence effects of dapagliflozin in T2D, it is unclear whether dapagliflozin has the same or similar effects in patients without T2D. Additional studies are necessary to validate the generalizability of the results.

5. Conclusions

In summary, dapagliflozin ameliorates endothelial cell senescence by promoting eNOS activation and suppressing ROS via SIRT1 pathway activation in T2D. Further studies are warranted to determine whether dapagliflozin can delay vascular aging in patients without diabetes.

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Data availability

The authors declare that all data presented in this study will be presented upon request from the corresponding author.

Consent for publication

All authors approved the manuscript for publication.

Author contribution statement

Shi Tai: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper; Contributed reagents, materials, analysis tools or data.

Ying Zhou, Liyao Fu, Huiqing Ding, Yuying Zhou, Zhiyi Yin, and Rukai Yang: Performed the experiments.

Ying Zhou, Liyao Fu, and Rukai Yang: Analyzed and interpreted the data.

Huiqing Ding, Yuying Zhou, and Zhiyi Yin: Contributed reagents, materials, analysis tools or data.

Zhenjiang Liu: Wrote the paper; Performed the experiments.

Shenghua Zhou and Zhenjiang Liu: Conceived and designed the experiments; Analyzed and interpreted the data.

Institutional review board statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the Ethics Committee of the Second Xiangya Hospital of Central South University, Changsha, China (Approval no. 2018.200) and in line with the Guide for the Care and Use of Laboratory Animals, NIH publication, 8th edition, 2011.

Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19152.

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