RESEARCH

SRT2104 attenuates diabetes-induced aortic endothelial dysfunction via inhibition of P53

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Abstract

Endothelial dysfunction contributes to diabetic macrovascular complications. Sirtuin 1 (SIRT1) protects against diabetic vasculopathy. SRT2104 is a novel SIRT1 activator and was not previously studied for its effects on diabetes-induced aortic endothelial dysfunction. Additionally, whether or to what extent deacetylation of P53, a substrate of SIRT1, is required for the effects of SIRT1 activation was unclear, given the fact that SIRT1 has multiple targets. Moreover, little was known about the pathogenic role of P53 in diabetes-induced aortic injury. To these ends, diabetes was induced by streptozotocin in C57BL/6 mice. The diabetic mice developed enhanced aortic contractility, oxidative stress, inflammation, P53 hyperacetylation and a remarkable decrease in SIRT1 protein, the effects of which were rescued by SRT2104. In HG-treated endothelial cells (ECs), P53 siRNA and SRT2104 produced similar effects on the induction of SIRT1 and the inhibition of P53 acetylation, oxidative stress and inflammation. Interestingly, SRT2104 failed to further enhance these effects in the presence of P53 siRNA. Moreover, P53 activation by nutlin3a completely abolished SRT2104's protection against HG-induced oxidative stress and inflammation. Further, forced activation of P53 by nutlin3a increased aortic contractility in the healthy mice and generated endothelial oxidative stress and inflammation in both the normal glucose-cultured ECs and the aortas of the healthy mice. Collectively, the present study demonstrates that P53 deacetylation predominantly mediates SRT2104's protection against diabetes-induced aortic endothelial dysfunction and highlights the pathogenic role of P53 in aortic endothelial dysfunction.

Key Words
- aorta
- diabetes
- endothelium
- P53
- SIRT1

Introduction

Macrovascular complications develop in >50% of the diabetic patients and result in high mortality (King & Wakasaki 1999, Forbes et al. 2004). Given the dramatic increase in the prevalence of diabetes mellitus (DM) (Giovannini et al. 2016, Meek & Morton 2016), it is of great importance and urgency to develop more effective approaches to prevent the pathogenesis of diabetic vasculopathy.
Previous reports highlighted the beneficial effect of sirtuin 1 (SIRT1) activation in diabetic vasculopathy (Csizsar et al. 2009, Orimo et al. 2009, Yang et al. 2011, Liu et al. 2016). Activation of SIRT1 by resveratrol improved palmitate-induced inflammation and insulin resistance (Liu et al. 2016), ameliorated HG-induced impairment in human umbilical vein endothelial cells (HUVECs) (Yang et al. 2011), induced mitochondrial biogenesis in aortic endothelial cells (ECs) of db/db mice (Csizsar et al. 2009) and improved aortic dysfunction in diabetic mice (Orimo et al. 2009). It is noted that resveratrol only increased SIRT1 protein expression by ~0.5- to 1.5-fold according to the previous reports (Csizsar et al. 2009, Yang et al. 2011, Liu et al. 2016). Given the promising effects of resveratrol, it is encouraging to develop more potent and more specific SIRT1 activators (Camins et al. 2010), which might provide a greater protection on diabetic vasculopathy.

SRT2104 is a novel, first-in-class, highly selective small-molecule activator of SIRT1 (Hoffmann et al. 2013) and was demonstrated to be safe in animals and humans (Libri et al. 2012, Hoffmann et al. 2013, Venkatasubramanian et al. 2013, 2016, Baksi et al. 2014, van der Meer et al. 2015). Because SRT2104 is a newly developed small molecule, to date, very limited studies have been performed on SRT2104 for its biological effects. It was previously reported for the effect of SRT2104 on DM-induced aortic endothelial dysfunction, which is the critical first step of diabetic vascular complications, with increased oxidative stress and inflammation as major contributors (Basta et al. 2004, 2005, Hulsmans & Holvoet 2010, Mittal et al. 2014, Sharma et al. 2017).

SIRT1 is a histone deacetylase that has multiple targets (Takata & Ishikawa 2003, Yeung et al. 2004, Rodgers et al. 2005, Sun et al. 2007, Canto et al. 2009, Wilson et al. 2010), among which P53 is known to be an important substrate for SIRT1 (Vaziri et al. 2001, Cheng et al. 2003, Kim et al. 2007). P53 is negatively regulated by mouse double minute 2 (MDM2) (Oliner et al. 1993), which sequesters and restricts P53 from exerting biological functions (Bargonetti & Manfredi 2002) and facilitates proteasomal degradation of P53 (Brooks & Gu 2006). Acetylation of P53 is essential for its stabilization and function (Brooks & Gu 2003). SIRT1 deacetylates P53 and thereby inactivates P53-triggered transcription of its downstream genes (Vaziri et al. 2001, Cheng et al. 2003, Kim et al. 2007). Although P53 was reported to participate in the pathogenesis of DM and a few of its complications (Deshpande et al. 2013, Tang et al. 2014, Kung & Murphy 2016, Li et al. 2016, Su et al. 2017), little was known for its role in diabetic vasculopathy. The pioneer work by Orimo and coworkers showed that high glucose (HG) resulted in hyperacetylation of P53 in HUVECs, the effect of which led to endothelial dysfunction (Orimo et al. 2009). These effects were reversed by resveratrol-induced SIRT1 activation (Orimo et al. 2009). However, it was still unknown whether or to what extent P53 deacetylation is required for the action of resveratrol, given that SIRT1 has multiple targets for deacetylation (Takata & Ishikawa 2003, Yeung et al. 2004, Rodgers et al. 2005, Sun et al. 2007, Canto et al. 2009, Wilson et al. 2010). It is also needed to further explore the pathogenic role of P53 in aortic injury, under both diabetic and non-diabetic conditions.

In summary, the present study aimed to test the effect of SRT2104 on DM-induced aortic injury in streptozotocin (STZ)-induced diabetic mice and HG-treated aortic ECs. In addition, P53 deacetylation was tested for its requirement in SRT2104’s action in HG-treated ECs. Furthermore, in order to define the role of P53 in endothelial dysfunction, P53 was activated by the specific MDM2 inhibitor nutlin3a, in both normal glucose (NG)-treated ECs and healthy mice.

### Materials and methods

#### Animal housing and experiments

CS7BL/6 mice were housed in the Animal Center of Jilin University at 22°C, on a 12:12-h light-dark cycle, with free access to rodent feed and tap water. All the experimental procedures were approved by the Institutional Animal Care and Use Committee at Jilin University and were in line with the NIH Guide for the Care and Use of Laboratory Animals. Either STZ (50 mg/kg/day, dissolved in 0.1 M sodium citrate, pH 4.5; Sigma-Aldrich) or sodium citrate were intraperitoneally injected to the 8-week-old male mice once every day, for 5 consecutive days (Zheng et al. 2011, Kato et al. 2016, Wu et al. 2016, Sun et al. 2017). Fasting glucose levels (4-h fast) were determined 1 week after the last injection. Mice with fasting glucose levels above 13.89 mM were considered diabetic.

To test the effect of SRT2104, mice were fed a standard diet (Xietong Organism, Nanjing, Jiangsu, PRC), or the standard diet supplemented with SRT2104 (MedChem Express, Shanghai, PRC) right after the confirmation of DM. SRT2104 was added to the diet at a dose of 1.33 g drug per kg of chow, formulated to provide daily doses of ~100 mg/kg, as previously described (Mercken et al. 2014).
Blood glucose was recorded on days 0, 140, 147, 154, 161 and 168, post DM onset.

To explore the pathogenic effect of P53 on aortic injury, 8-week-old healthy male mice were intraperitoneally injected with the specific MDM2 inhibitor nutlin3a (MedChem Express, 10 mg/kg) or vehicle (50% DMSO; Sigma-Aldrich), every other day, for a period of 4 weeks (Allam et al. 2011, Saito et al. 2016). The dose of nutlin3a at 10 mg/kg was a slight modification of the previously studied dose (Allam et al. 2011, Saito et al. 2016), in order to avoid severe glomerular sclerosis and high mortality (Saito et al. 2016).

At the end of the procedures, all the mice were killed under anesthesia by an intraperitoneal injection of chloral hydrate at 0.3 mg/kg (Pan et al. 2017) and their aortas harvested for analysis.

**Vascular contractility**

Enhanced vascular contractility is one of the key features of diabetic endothelial dysfunction (Sharma et al. 2017). Thus, the contractility of the thoracic aorta in response to phenylephrine (PE) was measured. Briefly, the thoracic aortas were cut into 4 mm segments and mounted on two L-shaped metal prongs. One prong was linked to a force-displacement transducer for continuous recording of isometric tension and the other was connected to a displacement device, which allowed adjustment of the distance between the two parallel prongs. The aortas were equilibrated for 45 min and normalized at 0, 15 and 25 mN to obtain the final micrometer setting between the prongs (Sharma et al. 2017). The aortas were then subjected to an oxygenated and pre-warmed high potassium physiological salt solution (KPSS, at 37°C), containing KCl 123 mM, MgSO\(_4\)·7H\(_2\)O 1.17 mM, NaHCO\(_3\) 25 mM, KH\(_2\)PO\(_4\) 1.18 mM, CaCl\(_2\) 2.5 mM, glucose 6.05 mM and EDTA 0.03 mM (Sharma et al. 2017), to determine the viability of the aortas. Vascular contraction in response to PE (at 10\(^{-9}\), 10\(^{-8}\), 10\(^{-7}\), 10\(^{-6}\), 10\(^{-5}\) and 10\(^{-4}\)M) was recorded.

**Morphological analysis**

The freshly harvested aortic tissues were fixed immediately into 10% buffered formalin solution and were embedded in paraffin, followed by sectioning into 5-µm-thick sections onto glass slides. Hematoxylin and eosin (H&E) staining was performed to evaluate the pathological features. Selection of areas to photograph and scoring was done by people blind to the identity of the samples.

**Immunohistochemical (IHC) staining**

IHC staining was done as previously described (Wu et al. 2014), using antibodies against 3-nitrotyrosine (3-NT, Millipore; 1:100), 4-hydroxynonenal (4-HNE, Alpha Diagnostic Int., San Antonio, TX, USA; 1:100), vascular cell adhesion molecule-1 (VCAM-1, Santa Cruz Biotechnology; 1:100), intercellular adhesion molecule-1 (ICAM-1, Santa Cruz Biotechnology; 1:100), SIRT1 (Abcam; 1:100) and acetylated P53 (Ac-P53, Abcam; 1:100). IHC-positive area was quantified within the full-thickness of the artery wall, including endothelium, tunica media and tunica externa. Selection of areas to photograph and scoring was done by people blind to the identity of the samples.

**Cell culture and experiments**

ECs were isolated from the aortas of 8-week-old C57BL/6 male mice, following the instructions by the established method for aortic EC isolation in mouse (Kobayashi et al. 2005). Briefly, each mouse was anesthetized by an intraperitoneal injection of chloral hydrate at 0.3 mg/kg (Pan et al. 2017), followed by the exposure of the abdominal aorta, which was then cut at the middle to release the blood. The aorta was perfused from the left ventricle, with PBS containing 1000 U/mL heparin and was then dissected out from the aortic arch to the abdominal aorta, and immersed in 20% fetal bovine serum (FBS; Gibco)-Dulbecco’s Modified Eagle Medium (DMEM; NG at 1 g/L; Gibco) containing 1000 U/mL heparin. The fat and connecting tissue were quickly removed under a stereoscopic microscope. The inside of the lumen was washed with serum-free DMEM (NG at 1 g/L), filled with collagenase II (2 mg/ml) and incubated at 37°C, for 45 min. ECs were removed by flushing with 5 ml 20% FBS-DMEM (NG at 1 g/L) and collected by centrifuging at 125 g, for 5 min. The cells were then suspended gently by pipette with 20% FBS-DMEM (NG at 1 g/L) and seeded in a collagen I-coated dish. After 7–10 days, confluent ECs were observable. After 2–3 passages, ECs were maintained in 10% FBS-DMEM (NG at 1 g/L).

To determine the effect of SRT2104 on HG-induced aortic endothelial oxidative stress and inflammation, ECs were treated with 10% FBS-DMEM (HG at 4.5 g/L), in the presence of SRT2104 (MedChem Express) at 3 µM (Mercken et al. 2014), for 48 h. To test whether or to what extent P53 deacetylation is required for SRT2104’s function, HG-treated ECs were subjected to a P53 siRNA (20 nM (Hirano et al. 2015)), its negative control (GenePharma, Suzhou, Jiangsu, PRC), in the presence or
absence of SRT2104, for 48 h. HG-treated ECs were further treated with SRT2104 (3 µM) and nutlin3a (1 µM (Allam et al. 2011), MedChem Express) in combination, with the aim of defining the role of P53 deacetylation in SRT2104’s action. In order to test the pathogenic effect of P53, NG (1 g/L)-cultured ECs were treated with nutlin3a at 1 µM, for 48 h. The transfection reagent RFect was provided by Changzhou Bio-generating Biotechnologies, Changzhou, Jiangsu, PRC.

**Real-time PCR**

Aortic tissue or cell lysates were used for quantitative real-time PCR, the procedure of which was described previously (Wu et al. 2014). The primers for glyceraldehyde 3-phosphate dehydrogenase (Gapdh), Icam-1, monocyte chemoattractant protein 1 (Mcp-1), P53 and Vcam-1 were purchased from Life Technologies.

**Western blot**

Western blot analysis was performed using cell lysates, as described in our previous study (Wu et al. 2014). Briefly, proteins were collected from cell lysates, by centrifuging at 12,000 g at 4°C for 15 min, with concentration measured using Bradford assay. After diluting in loading buffer and heating at 95°C for 5 min, the samples were subjected to electrophoresis on SDS-PAGE gel at 120 V. After electrophoresis, the proteins were transferred to nitrocellulose membranes, blocked in blocking buffer (5% milk and 0.5% BSA) for 1 h and washed 3 times with Tris-buffered saline containing 0.05% Tween 20 (Beyotime Biotechnology, Shanghai, PRC). The membranes were incubated with primary antibodies overnight, washed as aforementioned and reacted with secondary horseradish peroxidase-conjugated antibodies at room temperature for 1 h. The primary antibodies used were anti-Ac-P53 (Abcam; 1:500), anti-GAPDH (Santa Cruz Biotechnology, 1:3000), anti-ICAM-1 (Santa Cruz Biotechnology; 1:500), anti-P53 (Cell Signaling Technology; 1:1000) and anti-SIRT1 (Abcam; 1:1000).

**Quantitative analysis of reactive oxygen species (ROS) and lipid peroxides**

ROS and malondialdehyde (MDA) levels were measured in cell lysates, by using a ROS assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, PRC) and a lipid peroxidation assay kit (Sigma-Aldrich) respectively, following the manufacturers’ instructions.

**Results**

**SRT2104 attenuated the DM-enhanced aortic contractility**

Blood glucose levels were determined in all the mice every 4 weeks post DM onset (Fig. 1A). The STZ-treated mice developed significantly higher blood glucose levels, as compared with Ctrl (Fig. 1A). SRT2104 had no significant impact on blood glucose levels in both the diabetic and non-diabetic mice (Fig. 1A). To evaluate SRT2104’s effect on DM-induced aortic endothelial dysfunction, aortic contractility in response to PE was determined, in the presence or absence of SRT2104 (Fig. 1B). The diabetic mice had a remarkably increased aortic contractility in response to PE at 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ and 10⁻⁴M, as compared with Ctrl, the effect of which was significantly attenuated by SRT2104 (Fig. 1B). Morphological change was evaluated by the performance of H&E staining (Fig. 1C), which revealed no obvious morphological change, except for a mild increase in tunica media thickness and derangement of endothelial and smooth muscle cells in the aortas of the diabetic mice. This observation is in agreement with the previous findings in aortic morphology in mouse models of type 1 diabetes (Miao et al. 2013, Liu et al. 2014). SRT2104 blocked the DM-induced mild thickening of the tunica media (Fig. 1C), indicating the preventive effect of SRT2104 on DM-induced proliferation of smooth muscle cells.

**DM-induced aortic oxidative stress and inflammation were significantly attenuated by SRT2104**

To evaluate the effect of SRT2104 on DM-induced aortic oxidative stress and inflammation, in the following study, we performed IHC staining of the markers for oxidative stress and inflammation, in the following study, we performed IHC staining of the markers for oxidative stress and inflammation.
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SRT2104 elevated SIRT1 protein and inactivated P53 under both the diabetic and non-diabetic conditions

The effects of SRT2104 on SIRT1 expression and P53 activity were tested in the following study. As compared with Ctrl, the diabetic mice had a significantly lower expression of aortic SIRT1 (Fig. 3A) and developed P53 hyperacetylation (Fig. 3B). SRT2104 led to a significant increase (by 1.14-fold) in SIRT1 protein (Fig. 3A) and a marked decrease (by 54.3%) in Ac-P53 (Fig. 3B) in the non-diabetic mice. These effects were more remarkable in the diabetic mice (Fig. 3A and B). SIRT1 was increased by 3.79-fold and Ac-P53 was decreased by 82.6%. It is noted that the endothelium was the location where SIRT1 was preferably expressed under the non-diabetic condition and significantly less expressed under the diabetic condition (Fig. 3A). In contrast, the levels of Ac-P53 (Fig. 3B), oxidative stress (Fig. 2A and B) and inflammation (Fig. 2C and D) were low in the non-diabetic aortic endothelium and were markedly increased by DM. All these effects were reversed by SRT2104 (Figs 2A, B, C, D and 3A, B). These findings suggest that, upon DM, P53 hyperacetylation, oxidative stress and inflammation are induced in the aortic endothelium, which is a major battlefield where SIRT1 exerts its protection, possibly via P53 deacetylation. Thus, in the following steps, we researched the role of P53...
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deacetylation in SRT2104’s protection against HG-induced aortic endothelial oxidative stress and inflammation.

SRT2104 increased SIRT1 and blunted the HG-enhanced expression of Ac-P53 and markers for oxidative damage and inflammation

To further confirm the effect of SRT2104 in the aortic endothelium (Figs 2A, B, C, D, and 3A, B), ECs were treated with HG, in the presence or absence of SRT2104, for 48 h. SRT2104 reversed the HG-induced decrease in SIRT1 level (Fig. 4A), blocked the HG-enhanced Ac-P53 expression (Fig. 4B) and attenuated the HG-induced elevation of ROS, MDA (markers for oxidative stress; Fig. 4C) and mRNAs of Vcam-1 and Mcp-1 (markers for inflammation; Fig. 4D). These results in ECs confirm that the aortic endothelium is a target for SRT2104, with the alteration of P53 acetylation involved.

SRT2104 ameliorated HG-induced endothelial oxidative stress and inflammation through deacetylation of P53

Although P53 was suggested to be an important target of SIRT1 during diabetic vasculopathy (Orimo et al. 2009), it was still unknown whether or to what extent deacetylation of P53 is required for the protective effects of SIRT1. Therefore, we tested the effect of P53 silencing in the
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presence or absence of SRT2104 in HG-treated ECs. P53 siRNA resulted in a remarkable decrease in both the mRNA (Fig. 5A) and protein (Fig. 5B) levels of P53. P53 protein, but not the mRNA, was significantly decreased by SRT2104 (Fig. 5A and B). The capability of SRT2104 to decrease P53 protein level was lower than that of P53 siRNA (Fig. 5B).

Figure 3
SRT2104 elevated SIRT1 protein and inactivated P53 under both the diabetic and non-diabetic conditions. The protein expression of aortic (A) SIRT1 and (B) Ac-P53 was evaluated by IHC staining, with positive stained areas quantified. Data are normalized to Ctrl and presented as means ± s.d. (n=8).

*P<0.05 vs Ctrl; †P<0.05 vs DM. Bars: red, Ctrl; blue, Ctrl/SRT; green, DM; magenta, DM/SRT; Ac-P53, acetylated P53; SIRT1, sirtuin 1. A full colour version of this figure is available at https://doi.org/10.1530/JOE-17-0672.

Figure 4
SRT2104 increased SIRT1 and blunted the HG-enhanced expression of Ac-P53 and markers for oxidative damage and inflammation. The effect of SRT2104 was further evaluated in HG-treated ECs by determining protein levels of (A) SIRT1 and (B) Ac-P53 by Western blot, levels of (C) ROS and MDA by assay kits and (E) mRNA expression of Vcam-1 and Mcp-1 by RT-PCR. For Western blot and RT-PCR, GAPDH was used as loading control. Data are normalized to NG and presented as means ± s.d. (n=3). *P<0.05 vs NG; †P<0.05 vs HG. Bars: red, NG; blue, NG/mannitol; green, HG; magenta, HG/SRT; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HG, high glucose; MDA, malondialdehyde; Mcp-1, monocyte chemotactractive protein 1; NG, normal glucose; ROS, reactive oxygen species. A full colour version of this figure is available at https://doi.org/10.1530/JOE-17-0672.
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SRT2104 ameliorated HG-induced endothelial oxidative stress and inflammation through deacetylation of P53. To determine the role of P53 deacetylation in SRT2104’s effects, ECs were treated with HG and P53 siRNA, in the presence or absence of SRT2104. P53 (A) mRNA, (B) protein and (C) its acetylated form were determined. Additionally, (D) SIRT1 protein, (E) ROS and MDA levels, as well as (F) mRNA expression of Vcam-1 and Mcp-1 were measured in all the groups. The role of P53 deacetylation in mediating SRT2104’s protective effect was further tested by subjecting HG-treated ECs to a combination of SRT2104 and nutlin3a, with levels of (G) ROS, MDA and (H) mRNA expression of Vcam-1 and Mcp-1 determined.

Figure 5

Forced activation of P53 generated oxidative stress and inflammation under NG or non-diabetic conditions and caused aortic endothelial dysfunction in the healthy mice

In order to test the pathogenic role of P53, ECs were treated with NG, in the presence or absence of the specific MDM2 inhibitor, nutlin3a, for 48h. Nutlin3a elevated the protein levels of both P53 and ac-P53.
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Figure 6

Forced activation of P53 generated oxidative stress and inflammation under NG or non-diabetic conditions, and caused aortic endothelial dysfunction in the healthy mice. To test the pathogenic effect of P53, NG-cultured ECs and 8-week-old male C57BL/6 healthy mice were subjected to nutlin3a. (A) Protein levels of P53 and Ac-P53, (B) levels of ROS and MDA, as well as (C) mRNA expression of Vcam-1 and Mcp-1 were determined in NG-cultured ECs. In the healthy mice, (D) H&E staining was performed to evaluate aortic morphological change, and (E) IHC staining was performed to evaluate protein expression of Ac-P53, (F) 3-NT, 4-HNE, (G) VCAM-1 and ICAM-1. To further test aortic endothelial dysfunction, (H) vascular contraction was recorded in response to PE at $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$ and $10^{-4}$M. For Western blot and RT-PCR, GAPDH was used as loading control. For cell experiments, data are normalized to NG and presented as means ± s.d. ($n=3$). For animal experiments, data are normalized to Ctrl and presented as means ± s.d. ($n=8$). *$P<0.05$ vs NG; †$P<0.05$ vs Ctrl. Bars: red, NG; blue, NG/DMSO; green, NG/nutlin3a; magenta, Ctrl; orange, Ctrl/nutlin3a; NG, normal glucose. A full colour version of this figure is available at https://doi.org/10.1530/JOE-17-0672.

(Fig. 6A) and increased the levels of ROS, MDA (Fig. 6B) and the mRNA levels of Vcam-1 and Mcp-1 (Fig. 6C). Further investigations were performed to test the effect of forced activation of P53 by nutlin3a on the aortas of the healthy mice. No significant difference in aortic histology, as revealed by H&E staining (Fig. 6D), was observed between the Ctrl mice and the nutlin3a-treated mice, except for a slight derangement of endothelial
and smooth muscle cells (Fig. 6D). The nutlin3a-treated mice increased aortic Ac-P53 (Fig. 6D and E) and enhanced aortic oxidative damage (Fig. 6D and F) and inflammation (Fig. 6D and G). In order to further test the effect of P53 activation on endothelial dysfunction, aortic contraction was determined in the aortas of both the Ctrl mice and the nutlin3a-treated mice, in response to PE. Nutlin3a markedly enhanced aortic contraction in the presence of PE at $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$ and $10^{-4}$M, demonstrating that P53 contributes to endothelial dysfunction under non-diabetic condition.

**Discussion**

The present study researched the protective effect of the novel SIRT1 activator, SRT2104, on DM/HG-induced aortic endothelial dysfunction. Decreased SIRT1 protein, hyperacetylated P53 and enhanced endothelial injury were found in the aortas of the diabetic mice and HG-treated ECs, the effects of which were rescued by SRT2104. As compared with SRT2104, P53 gene silencing led to a similar extent of reduction in Ac-P53 level, induction of SIRT1 protein and decrease in oxidative stress and inflammation in HG-treated ECs. Interestingly, SRT2104 did not further attenuate HG-induced oxidative stress and inflammation in ECs upon P53 gene silencing, nor did it produce such protection when P53 was activated, indicating the requirement of P53 deacetylation in SRT2104’s action. P53 activation by nutlin3a generated aortic endothelial oxidative stress and inflammation under both NG and non-diabetic conditions and induced aortic endothelial dysfunction in the healthy mice, establishing the pathogenic role of P53 in the aorta.

SIRT1 activation is proven beneficial for diabetic vasculopathy (Csiszar et al. 2009, Orimo et al. 2009, Yang et al. 2011, Liu et al. 2016). The known SIRT1 activator, resveratrol, has been intensively studied for its effects in diseases, including diabetic vasculopathy (Csiszar et al. 2009, Yang et al. 2011, Liu et al. 2016). According to these reports, resveratrol increased SIRT1 protein by $-0.5$- to 1.5-fold and produced promising effects (Csiszar et al. 2009, Yang et al. 2011, Liu et al. 2016). In contrast, SRT2104 was found, in the present study, to elevate SIRT1 protein level by 3.79-fold in the aortas of the diabetic mice (Fig. 3A) and 3.91-fold in HG-treated ECs (Fig. 4A), despite its less potent effect on the increase of SIRT1 protein in the aortas of the non-diabetic mice (1.14-fold, Fig. 3A). This discrepancy could be due to the difference in the basal Ac-P53 levels between the diabetic and the non-diabetic mice (Fig. 3B). P53 negatively regulates SIRT1, as demonstrated in the present study (Fig. 5D) and those by others (Yamakuchi & Lowenstein 2009, Audrito et al. 2011, Castro et al. 2013). Mechanistically, P53 transcriptionally activates microRNA-34a, which targets Sirt1 mRNA, leading to decreased SIRT1 protein. SIRT1, in turn, deacetylates P53 and thereby inhibits the transcription of miR-34a (Yamakuchi & Lowenstein 2009, Audrito et al. 2011, Castro et al. 2013). In the present study, the aortic Ac-P53 level was significantly higher in the diabetic mice compared to the non-diabetic mice (Fig. 3B), thus, may provide enough targets for the SRT2104-induced SIRT1 to function through. The effects of SRT2104, including the inhibition of P53 and the aortic oxidative stress and inflammation, could be amplified within the SIRT1/P53/miR-34a positive feedback loop (Yamakuchi & Lowenstein 2009), when it initially triggered SIRT1 activation.

In addition to the effect of SRT2104 on DM-induced aortic injury, another novel finding of the present study is the establishment of P53 as the main target that predominantly mediates the benefits of SRT2104-led SIRT1 activation, in spite of the various targets of SIRT1 for deacetylation. This is supported by the results that SRT2104 failed to further decrease Ac-P53 (Fig. 5C), increase SIRT1 (Fig. 5D) and attenuate oxidative stress and inflammation (Fig. 5E and F), and SRT2104’s protective effects were completely abolished upon P53 activation (Fig. 5G and H). Although previous studies indicated P53 to be an important target of SIRT1, it was unclear whether or to what extent P53 is required for SIRT1’s function. In the present study, by silencing P53, P53 was found to be the key player in this action (Fig. 5A, B, C, D, E, F, G and H). Despite the weaker effect of SRT2104 on the decrease of P53 protein level (Fig. 5B), SRT2104 resulted in a similar reduction of Ac-P53 as compared with P53 siRNA (Fig. 5C). Therefore, P53 siRNA and SRT2104 decrease Ac-P53 via different mechanisms. P53 siRNA directly lowers the amount of P53 protein, whereas SRT2104 relies more on its deacetylase activity. In the presence of P53 siRNA, co-treatment with SRT2104 did not produce a greater decrease in both P53 mRNA and protein (Fig. 5A and B). This might be caused by the strong inhibitory effect of P53 siRNA on P53 gene expression (Fig. 5A and B), the effect of which provided few P53 as targets for SIRT1.

One important finding of this study was the definition of the pathogenic role of P53 in the aorta. By activating P53 in NG-cultured ECs and in the aortas of the healthy mice,
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Abstract

we addressed the critical role of P53 in inducing aortic oxidative stress and inflammation. In fact, the pathogenic effects of P53 have been demonstrated in DM (Kung & Murphy 2016, Li et al. 2016) and its complications, such as diabetic cardiomyopathy (Raut et al. 2016), nephropathy (Menini et al. 2007) and peripheral artery disease (Morimoto et al. 2011). Given the deleterious effects of P53 in DM and its multiple complications, P53 inhibition could be a promising strategy, which may benefit multiple organs in diabetic patients.

In the present study, SRT2104 was found to prevent HG-induced aortic endothelial injury, the effect of which was almost completely mediated by deacetylation of P53 (Fig. 5C, D, E and F). As compared with SRT2104, inhibition of P53 by P53 siRNA led to a similar extent of increase in SIRT1 protein level, as well as protection against endothelial oxidative stress and inflammation (Fig. 5D, E and F). These findings suggest an intact inhibitory crosstalk between SIRT1 and P53 and might indicate both SIRT1 activation and P53 gene silencing to be effective approaches to attenuate DM-induced aortic endothelial injury.

A possible concern for inhibition of P53, the guardian of the genome in coordinating cellular responses to genotoxic stress (Lane 1992, Levine 1997), could be tumor generation. We speculate that a basal level of P53 must be maintained in order to avoid tumor generation. In the present study, SRT2104 at 100mg/kg decreased and maintained aortic Ac-P53 of the diabetic mice at a level in parallel to Ctrl (Fig. 3B). This did not induce tumors in the diabetic mice, nor in the non-diabetic mice in which the aortic Ac-P53 level was 45.7% of that of the Ctrl (Fig. 3B). Moreover, in the finished clinical trials of SRT2104 on T2DM (Baksi et al. 2014), lipopolysaccharide-induced inflammation (van der Meer et al. 2015) and cardiovascular function in otherwise healthy cigarette smokers (Venkatasubramanian et al. 2013), SRT2104 was demonstrated to be safe and well tolerated at the higher dose 2.0g per day, for 28 days. Nonetheless, special attention should be paid to the level of P53 during P53 inhibition or SIRT1 activation in future studies, including clinical trials.

Only a limited number of clinical trials were performed on the effects of SRT2104 in diseases, among which SRT2104’s effects on endothelial function were studied in people with T2DM (Venkatasubramanian et al. 2013) and in otherwise healthy cigarette smokers (Noh et al. 2017). Interestingly, SRT2104 did not have a significant impact on endothelial function in these two clinical trials. It is noted that SRT2104 was given for only 28 days in total, which may produce little protection. Hence, a longer period of SRT2104 treatment, such as a 24-week treatment in mice as tested in the present study, would lead to more remarkable effects. Moreover, vasodilators were used to study the effect of SRT2104 on endothelial dysfunction in these clinical trials, the results of which may differ from a vasoconstrictor like PE used in the present study. Furthermore, treatment with SRT2104 at much earlier stages of diabetes or smoking may have much better outcomes on endothelial function, since diabetic complications are almost impossible to be reversed once they develop into advanced stages. Therefore, the effect of SRT2104 on endothelial function should be tested in future clinical trials with larger sample numbers and longer treatment and follow-up periods, with the initiation time of treatment and methods of evaluation carefully considered and selected.

Taken together, here, we report the protective effect of the novel SIRT1 activator SRT2104 against DM-induced aortic endothelial dysfunction. These beneficial effects were predominantly mediated by P53 deacetylation. Further, P53 was defined as a key pathogenic factor in aortic endothelial dysfunction. These findings may provide a basis for the understanding of the actions of SIRT1 and P53 in the aorta and might bring new insights into the management of diabetic vasculopathy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported in part by the National Natural Science Foundation of China (81600573) and Norman Bethune Program of Jilin University (2015438) to Hao Wu and Department of Science and Technology of Jilin Province (20170204032YY) and Development and Reform Commission of Jilin Province (2014G072) to Junnan Wang and Research Program of Jilin University (SR2173203428) to Ye Jia.

Author contribution statement

Hao Wu conceived the project. Hao Wu, Junnan Wang and Ye Jia designed the experiments. Hao Wu, Junduo Wu, Shengzhu Zhou, Wenlin Huang, Ying Li and Huan Zhang researched and interpreted the data. Hao Wu, Junnan Wang and Ye Jia wrote the manuscript. Hao Wu, Junduo Wu, Shengzhu Zhou, Wenlin Huang, Ying Li, Huan Zhang, Junnan Wang and
Ye Jia stimulated discussion, reviewed and revised the manuscript. Hao Wu, Junnan Wang and Ye Jia provided funding for the research. Junnan Wang and Ye Jia are guarantors of this work. All the authors approve the version to be published.

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Received in final form 3 January 2018
Accepted 25 January 2018
Accepted Preprint published online 25 January 2018