RESEARCH

SRT1720 retards renal fibrosis via inhibition of HIF1A/GLUT1 in diabetic nephropathy

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Abstract

Renal fibrosis is the major pathological characteristic of diabetic nephropathy (DN). Reportedly, increased SIRT1 expression played a renal protective role in animal models of DN. This study was designed to elucidate the molecular mechanisms underlying the protective effects of SRT1720, an SIRT1 activator, against diabetes-induced renal fibrosis. Type 2 diabetic mice (db/db) were treated with SRT1720 (50 mg/kg/day) by gavage for 10 weeks. Renal proximal tubular epithelial cells (HK-2 cells) were treated with high glucose (HG, 30 mM) in the presence or absence of SRT1720 (2.5 μ M) for 48 h. We observed that impaired SIRT1 expression and activity were restored by SRT1720 administration in db/db mice as well as in HG-treated HK-2 cells. Moreover, SRT1720 administration improved the renal function, attenuated glomerular hypertrophy, mesangial expansion, glomerulosclerosis and interstitial fibrosis and inhibited TGFB1 and CTGF expressions and nuclear factor κ B (NF- κ B) activation in db/db mice. Similarly, HG-induced epithelial-to-mesenchymal transformation (EMT) and collagen IV and fibronectin expressions were inhibited in SRT1720-treated HK-2 cells. Mechanistic studies demonstrated that SRT1720 suppressed HIF1A, GLUT1 and SNAIL expressions both *in vivo* and *in vitro*. Furthermore, *HIF1A* or *GLUT1* knockdown effectively abrogated HG-induced EMT and collagen IV and fibronectin expressions in HK-2 cells. These findings suggest that SRT1720 prevented diabetes-induced renal fibrosis via the SIRT1/HIF1A/GLUT1/SNAIL pathway.

Key Words

- ▶ diabetic nephropathy
- ▶ SRT1720
- ▶ renal fibrosis
- ▶ HIF1A
- ▶ epithelial to mesenchymal transition

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Introduction

Diabetic nephropathy (DN) is a microvascular complication of diabetes mellitus, accounting for almost 50% of all end-stage renal disease cases. Renal fibrosis is a major characteristic of DN and an important predictor of renal dysfunction (Gilbert & Cooper 1999). However, the molecular mechanisms by which diabetes induced renal fibrosis remain largely unknown.

Silent mating type information regulation 2 homolog 1 (SIRT1) is a member of the Sirtuin family (SIRT 1–7). Reportedly, SIRT1 alleviated diabetes by preventing hepatic insulin resistance and enhancing pancreatic insulin secretion (Moynihan *et al.* 2005, Wang *et al.* 2011). In addition, protective effects of SIRT1 on renal injuries caused by ischaemia/reperfusion (I/R), unilateral ureter

obstruction (UUO) or aldosterone have been reported (Li *et al.* 2010, Yuan *et al.* 2012, Fan *et al.* 2013). Resveratrol, an SIRT1 activator, prevented DN in db/db mice via SIRT1-PGC1A signalling (Kim *et al.* 2013). Moreover, podocyte-specific *Sirt1* overexpression attenuated diabetes-induced podocyte loss and reduced oxidative stress in the glomeruli of OVE26 mice (Hong *et al.* 2018). Similarly, heterogeneous nuclear ribonucleoprotein F protected kidneys from oxidative stress and nephropathy by stimulation of SIRT1 expression in db/db mice (Lo *et al.* 2017). Therefore, stimulation of SIRT1 expression is a therapeutic target to prevent the progression of renal injury in diabetes.

SRT1720 is a small molecule that dramatically increases SIRT1 deacetylase activity (Milne *et al.* 2007). SRT1720 induced significant renal SIRT1 mRNA and protein expressions in cholic acid-fed mice (Kulkarni *et al.* 2016) and markedly improved renal tubular pathology and overall renal function in adult mice following I/R (Fan *et al.* 2013). Our recent study has shown that SRT1720 attenuated renal fibrosis by inhibiting connective tissue growth factor (CTGF) expression and reducing oxidative stress in UUO mice (Ren *et al.* 2017). However, effects of SRT1720 on DN remain unknown.

Chronic hypoxia has been implicated in the development and progression of DN (Singh *et al.* 2008). Hypoxia inducible factor 1 alpha (HIF1A) is a key transcriptional regulator of cellular adaptation to hypoxia. HIF1A stimulated expression of various gene products in hypoxia (such as TWIST and SNAIL), promoting renal fibrosis and chronic kidney disease (CKD) progression in certain models (Higgins *et al.* 2007, Kimura *et al.* 2008, Sun *et al.* 2009, Du *et al.* 2014). Nayak *et al.* have demonstrated that HIF1A mediated renal fibrosis in type 1 diabetic mice (Nayak *et al.* 2016). In hypoxia, SIRT1 deacetylated and inactivated HIF1A, inhibiting fibrosis in hypoxic adipose tissue (Li *et al.* 2016). However, whether SIRT1 alleviates renal fibrosis via HIF1A inhibition remains unknown in DN. Therefore, the aims of the present study were to determine whether SRT1720 exerts a beneficial effect on renal fibrosis in DN and to explore the underlying signalling pathway.

Materials and methods

Antibodies and other reagents

SRT1720 was purchased from BioChemPartner (Shanghai, China). Antibodies against SIRT1, HIF1A, GLUT1, fibronectin, collagen IV, E-cadherin, α -SMA, NF-KB p65 and

8-hydroxydeoxyguanosine (8-OHdG) were obtained from Abcam. Antibodies for transforming growth factor beta 1 (TGF β 1), NAD(P)H oxidase 4 (NOX4), CTGF, SNAIL, Histone H3 and β -actin were purchased from Proteintech (Chicago, USA). Goat anti-rabbit or mouse IgG-HRP secondary antibodies, TRITC-labelled goat anti-rabbit secondary antibody and FITC-labelled goat anti-rabbit or mouse IgG secondary antibodies were purchased from Zhongshan Goldenbridge Biotechnology (Beijing, China). Biochemical parameters in urine and plasma were measured with the reagent kits purchased from BioSino Biotechnology and Science (Beijing, China). PVDF membranes were purchased from Millipore. All culture media were from Gibco-BRL, and the Lipofectamine RNAiMAX was purchased from Invitrogen Life Technologies.

Animals

Male C57BL/KsJ db/db mice and littermate control db/m mice were obtained from the Nanjing University. All experimental animals were housed under specific pathogen-free conditions with clean sawdust bedding according to the guidelines of the National Institute of Health. The Ethics Committee of Hebei Medical University reviewed and approved all procedures involving mice. At 10 weeks of age, the mice randomly divided the mice into three groups: db/m ($n=6$), db/db ($n=6$) and db/db+SRT1720 ($n=6$). The db/db+SRT1720 group received 50 mg/kg SRT1720 every day by gavage. SRT1720 dose was selected based on a previous publication (Kulkarni *et al.* 2016). Mice in the control or diabetes groups received the same amount of solvent (30% PEG400/0.5% Tween80/5% propylene glycol). At the age of 20 weeks, animals were killed and the kidneys were harvested for further analysis. Plasma and urinary supernatants were collected and stored at -80°C until further analysis.

Blood and urine chemistry

At the age of 20 weeks, mice were individually housed in metabolic cages to collect 24-h urine. Blood samples were withdrawn from the retrobulbar venous plexus using a capillary after 12-h fasting. Plasma and urinary supernatants were measured using reagent kits according to the manufacturer's instructions.

Urine 8-OHdG measurements

Urine specimens were centrifuged at 220g for 10 min to remove particulates. We used the supernatants and

measured 8-OHdG levels using a competitive *in vitro* enzyme-linked immunosorbent assay kit (Abcam) according to the manufacturer's instructions.

Tissue histology

The kidneys were fixed in 4% paraformaldehyde for 48 h, paraffin embedded and cut. The sections were stained with periodic acid-Schiff and Masson's Trichrome staining. A semi-quantitative index was used to evaluate the degree of glomerular mesangial expansion and sclerosis (Zhao *et al.* 2006).

Human renal biopsies

Human renal biopsies were collected in the Second Hospital of Shanxi Medical University. The protocol was approved by the Ethical Committees of Shanxi Medical University. A total of 22 kidney biopsies from individual patients in our analyses: 18 samples were collected from patients with type 2 diabetes and biopsy-proven DN and 4 normal human renal tissue samples were obtained from distant portions of kidneys surgically excised because of the presence of localised neoplasms. In the DN group, renal biopsies were performed to exclude the coexistence of other types of kidney disease.

Immunohistochemistry

After formalin fixation and paraffin embedding, 4 µm sections were deparaffinised with xylene and rehydrated by passing them through graded ethanol. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide in 100% methanol for 15 min after antigen retrieval with heat in 10 mM citrate buffer. Next, 10% normal goat serum in phosphate-buffered saline (PBS) were added to the sections for 30 min at room temperature to block nonspecific antibody binding. Then sections were incubated with the following primary antibodies overnight at 4°C in PBS: SIRT1, HIF1A, TGFβ1, E-cadherin, α-SMA, SNAIL, CTGF, NF-κBp65, NOX4 and 8-OHdG. After rinsing in PBS, sections were incubated with biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin. Labels were visualised with diaminobenzidine (DAB) to produce a brown colour, and sections were counterstained with hematoxylin.

Cell culture

HK-2 cells (ATCC, American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in 95% air, 5% CO₂ atmosphere in DMEM plus DMEM-F12 medium (1:1) containing 10% foetal bovine serum and 1% streptomycin-penicillin mixture. Depending on different experiments, HK-2 cells were pre-treated with serum-free medium for 24 h, and then stimulated them with 5.6 mM glucose plus 24.4 mM mannitol (Control), HG (30 mM glucose) or HG plus SRT1720 (2.5 µM) at indicated time points. Moreover, siRNAs were transfected into HK-2 cells. The siRNA targeting human *HIF1A* (5'-CGAGGAAGAACUAUGAACATT-3'), *GLUT1* (5'-CCAAGAGUGUGCUAAAAGAATT-3') or negative control (5'-UUCUCCGAACGUGUCACGUTT-3') were synthesised by Genepharma (Suzhou, China).

Immunofluorescence assay

Expressions of fibronectin, collagen IV and GLUT1 in renal tissues were detected using immunofluorescence assays. Frozen renal tissue sections were fixed with 4% paraformaldehyde at room temperature for 10 min and permeabilised in 0.2% Triton X-100 for 10 min. After 30-min blocking with goat serum at 37°C, renal tissues were incubated with primary antibodies in PBS overnight at 4°C. After washing, renal tissues were incubated with TRITC/FITC-labelled secondary antibodies for 1 h at 37°C. Images for fibronectin and GLUT1 were captured at 40× magnification using a fluorescence microscope (model No. BX63, Olympus). Images of collagen IV were obtained from a Leica DM2500 confocal microscope and quantitated using Image-Pro Plus 6.0 (Media Cybernetics).

Cells cultured in six-well chamber slides were fixed with 4% paraformaldehyde for 40 min at 4°C and permeabilised with 0.2% Triton X-100 for 10 min at 37°C. After washing, cells were incubated with antibodies against SIRT1, HIF1A, GLUT1, fibronectin, collagen IV, E-cadherin or α-SMA overnight at 4°C. Then, cells were incubated with the FITC-labelled secondary antibody (1:150) for 1 h at 37°C. After washing with PBS, cell nuclei were stained with DAPI for 10 min. Images were obtained using a Leica DM2500 confocal microscope.

Western blot analyses

Kidney tissues and cultured cells were homogenized in a lysis buffer (Millipore) according to the standard

procedures. Nuclear proteins were extracted from mouse kidneys using a commercial nuclear extraction kit (Active Motif, Carlsbad, CA, USA). Protein concentrations were determined using the bicinchoninic acid protein assay kit (Solarbio, Beijing, China). For immunoblotting, same amounts of protein (30–50 µg) were electrophoresed on SDS-PAGE and transferred onto PVDF membranes. Blots were incubated with primary antibodies (diluted to 1:1000 for SIRT1, HIF1A, GLUT1, SNAIL, fibronectin, collagen IV, E-cadherin, α -SMA, TGF β 1, CTGF, NOX4, NF- κ Bp65, β -actin and Histone H3 antibodies) overnight at 4°C, and then with goat anti-rabbit or mouse IgG horseradish peroxidase conjugates, and finally scanned them using the GE-Amersham Imager 600 (General Electric Company, USA). Densitometry of the bands was performed using the NIH Image J 1.50 software.

Immunocytochemistry

HK-2 cells were fixed with 4% paraformaldehyde and permeabilised with 0.2% Triton-X 100 for 10 min at 37°C. After rinsing with PBS, cells were incubated with antibody against SNAIL overnight at 4°C. Then, cells were incubated with biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin. DAB was used to produce brown colour for visualisation.

SIRT1 deacetylase activity assay

SIRT1 activities in renal tissue and HK-2 cells were determined using a fluorometric SIRT1 assay kit (Sigma-Aldrich) (Li *et al.* 2017b). In brief, samples were incubated with 10 µL SIRT1 substrate solution in the presence or absence of NAD⁺. Samples were incubated at 37°C for 1 h. Next, 5 µL of developing buffer was added to each well, samples were incubated at 37°C for 10 min. The fluorescence intensities were measured using a microplate fluorometre (excitation wavelength=360 nm, emission wavelength=450 nm). Each experiment was performed in triplicates ($n=6$).

Reactive oxygen species (ROS) measurement

Mitochondrial ROS were detected using the fluorescence MitoSOX red probe (Invitrogen). Cells were incubated in 5 µM of MitoSOX Red reagent working solution at 37°C for 10 min and then washed three times with warm buffer. Fluorescence intensity was observed under a Leica DM2500 confocal microscope. Each experiment was performed in triplicate ($n=6$).

Statistical analysis

Values are expressed as mean $\pm$ standard error of means (S.E.M.). Statistical differences were assessed using one-way ANOVA for multiple groups and Bonferroni tests were carried out to determine the significant differences in multiple comparisons. All analyses were performed using the SPSS 21.0 statistical software package (SPSS). $P<0.05$ was considered significant.

Results

Animal characteristics

As shown in Table 1, db/db mice showed significant increase in blood glucose level and body weight compared with db/m mice. SRT1720 treatment attenuated this increase in blood glucose level and body weight of db/db mice. The 24-h urine albumin excretion, urine albumin/creatinine ratio, serum creatinine and blood urea nitrogen values in db/db mice were significantly higher than those in db/m mice, which were alleviated by SRT1720 treatment.

SRT1720 increased renal SIRT1 expression and activity in db/db mice

Immunohistochemistry staining showed that SIRT1 was mainly expressed in glomeruli and renal tubules and that renal SIRT1 expression was reduced in db/db mice compared with that in db/m mice. SRT1720 treatment significantly increased renal SIRT1 expression in db/db mice (Fig. 1A). Western blot showed that diabetes was associated with decreased renal cortical SIRT1 expression and that this decrease was partially reversed by SRT1720 treatment (Fig. 1B). Similarly, SRT1720 treatment markedly increased renal SIRT1 activity in db/db mice (Fig. 1C). These results confirmed that SRT1720 regulates renal SIRT1 expression and activity in db/db mice.

SRT1720 prevented renal extracellular matrix (ECM) accumulation and fibrosis in db/db mice

The mesangial area in db/db mice kidneys was larger than that in db/m mice, and SRT1720 treatment significantly reduced this area (Fig. 2A and B). As depicted in the Masson Trichrome staining, increased interstitial fibrosis in the kidneys of db/db mice was observed compared with that in kidneys of db/m mice, and this increase

Table 1 Physical and metabolic parameters after completion of the study. Data are presented as the mean $\pm$ s.e.m. ($n = 6$).

Group	BG (mmol/L)	BW (g)	KW (mg)	BUN (mmol/L)	Scr (μ mol/L)	UAE (μ g/24h)	UACR (μ g/mg)
db/m	13.98 $\pm$ 0.89	25.74 $\pm$ 0.84	194.42 $\pm$ 18.57	7.67 $\pm$ 1.21	23.95 $\pm$ 3.45	5.71 $\pm$ 2.76	14.62 $\pm$ 2.41
db/db	35.85 $\pm$ 6.51*	60.35 $\pm$ 3.38*	217.97 $\pm$ 27.31	14.63 $\pm$ 3.27*	54.41 $\pm$ 9.07*	308.57 $\pm$ 17.14*	118.24 $\pm$ 13.52*
db/db + SRT	31.48 $\pm$ 7.64#	51.28 $\pm$ 3.58#	220.83 $\pm$ 22.33	7.99 $\pm$ 1.47#	34.66 $\pm$ 1.54#	226.67 $\pm$ 54.01#	65.78 $\pm$ 9.76#

* $P < 0.05$ vs db/m; # $P < 0.05$ vs db/db.

BG, blood glucose; BUN, blood urea nitrogen; BW, body weight; KW, kidney weight; Scr, serum creatinine; SRT, SRT1720; UACR, urine albumin/creatinine ratio; UAE, urine albumin excretion.

was reversed by SRT1720 treatment (Fig. 2A and C). Moreover, SRT1720 affected the expressions of renal fibrosis markers, collagen IV and fibronectin (Fig. 2A). As indicated by immunofluorescence, db/db mice exhibited increased collagen IV expression compared with db/m mice. However, this increased collagen IV expression was reduced by SRT1720 treatment (Fig. 2A and D). Moreover, SRT1720 showed inhibitory effect on increased fibronectin expression in the kidneys of db/db mice compared with that in untreated mice (Fig. 2A and E). These results demonstrated that SRT1720 can diminish ECM accumulation and fibrosis in the kidneys of db/db mice.

Effect of SRT1720 on renal expression of E-cadherin, α -SMA, HIF1A, GLUT1 and SNAIL in db/db mice

Epithelial marker E-cadherin and mesenchymal marker α -SMA were examined to determine effects of SRT1720 on renal EMT. As shown in immunohistochemistry, E-cadherin expression was lower but α -SMA expression was higher in the renal tubules of db/db mice than in those of db/m mice, and the trends were reversed by SRT1720 treatment (Fig. 3A). Western blotting corroborated these results (Fig. 3B).

To investigate mechanisms underlying the fibrosis inhibitory effects of SRT1720, expressions of HIF1A, GLUT1 and SNAIL were detected. As shown in Fig. 3C, HIF1A was expressed in the renal tubules and glomeruli.

Renal HIF1A expression was significantly higher in db/db mice than in db/m mice. Importantly, HIF1A expression in diabetic kidneys was inhibited by SRT1720 treatment. Similar results were observed in terms of GLUT1 and SNAIL expressions, which were mainly expressed in the renal tubules as shown in immunofluorescence and immunohistochemistry images (Fig. 3C). Western blot revealed that renal HIF1A, GLUT1 and SNAIL expressions were significantly higher in db/db mice than in db/m mice, but they were decreased after SRT1720 treatment (Fig. 3D). Together, these results demonstrated that SRT1720 conferred regulatory effect on EMT of tubular cells and expressions of HIF1A, GLUT1 and SNAIL in db/db mice.

SRT1720 inhibited renal TGF β 1, CTGF and NF- κ B expressions and reduced oxidative stress in db/db mice

TGF β 1, the most potent inducer of renal fibrosis in diabetes, modulates ECM protein expression either alone or together with CTGF (Okada *et al.* 2005). Renal TGF β 1 and CTGF expressions were significantly increased in db/db mice compared with those in db/m mice, and SRT1720 treatment suppressed this effect (Fig. 4A and B). A previous study has shown that NF- κ B activation is associated with inflammation and fibrosis in DN (Sun *et al.* 2016). NF- κ B was activated in diabetic kidney,

Figure 1 Effect of SRT1720 on SIRT1 expression and activity in db/db mice. (A) Immunohistochemical staining images of SIRT1 in kidney sections (scale bar, 20 μ m). Arrows indicate positive SIRT1 staining. (B) SIRT1 expression was assessed by Western blot in renal cortical homogenates. (C) SIRT1 activity. Data are expressed as mean $\pm$ s.e.m. ($n = 6$). * $P < 0.05$ vs db/m; # $P < 0.05$ vs db/db.

Figure 2

Effect of SRT1720 on renal histopathology. (A) Staining of kidney with periodic acid Schiff (PAS) and Masson Trichrome, and immunofluorescence staining for fibronectin and collagen IV (scale bar, 20 μ m). NC, immunofluorescence images obtained from three groups without primary antibody as a negative control. Mesangial index (B), tubulointerstitial injury index (C) and semi-quantitative analyses of collagen IV (D) were measured. (E) Fibronectin expression was detected using Western blot. Data are expressed as mean $\pm$ S.E.M. ($n = 6$). * $P < 0.05$ vs db/m; # $P < 0.05$ vs db/db.

and SRT1720 treatment significantly reduced the NF-KB transfer from the cytoplasm to nucleus in db/db mice compared with that in untreated mice (Fig. 4A and C). Furthermore, NOX4 expression in diabetic kidneys was evaluated. Increased NOX4 expression in diabetic kidneys was significantly attenuated by SRT1720 treatment (Fig. 4A and D). Moreover, levels of 8-OHdG, a marker of oxidative stress, were measured in renal tissues and urine. SRT1720 treatment lowered 8-OHdG levels in renal tissues and urine of db/db mice compared with those in renal tissues and urine of untreated mice (Fig. 4A and E).

SRT1720 blocked HG-induced EMT and ECM proteins expression in HK-2 cells

SIRT1 expression and activity were decreased in HG-treated HK-2 cells compared with those in control group. SRT1720 treatment stimulated SIRT1 expression and activity in HG-treated HK-2 cells (Fig. 5A, B and C). Effects of SRT1720 on HG-induced EMT and ECM protein expression in

HK-2 cells were investigated. Immunofluorescent staining and Western blot revealed that HG-induced HK-2 cells exhibited E-cadherin downregulation and α -SMA upregulation. However, SRT1720 treatment attenuated this HG-induced decrease in E-cadherin expression and increase in α -SMA expression (Fig. 5A and B). In control group, HK-2 cells exhibited a typical epithelial cuboidal shape, but HG treatment produced spindle-like shape. However, this HG-induced morphological change was reversed by SRT1720 treatment (Fig. 5D). In addition, fibronectin and collagen IV expressions dramatically increased in HG-treated HK-2 cells compared with those in control group. Exposure to SRT1720 significantly reduced HG-induced fibronectin and collagen IV expressions in HK-2 cells (Fig. 5E and F). Next, effects of SRT1720 on HG-induced mitochondrial ROS generation were evaluated in HK-2 cells. As shown in Fig. 5G, ROS generation was inhibited by SRT1720 treatment in HK-2 cells under HG conditions. These results indicated that SRT1720 exerts inhibitory effects on HG-induced EMT and ECM protein expression in HK-2 cells.

Figure 3

Effect of SRT1720 on renal expression of E-cadherin, α -SMA, HIF1A, GLUT1 and SNAIL in db/db mice. (A) Immunohistochemical staining of E-cadherin and α -SMA in kidney sections (scale bar, 100 μ m). (B) Expressions of E-cadherin and α -SMA were assessed using Western blot. (C) Representative kidney sections of HIF1A and SNAIL expression detected by immunohistochemistry staining and GLUT1 expression detected by immunofluorescence staining (scale bar, 20 μ m). Arrows indicate positive HIF1A staining. NC, immunohistochemistry images obtained from three group without primary antibody as a negative control. (D) Expressions of HIF1A, GLUT1 and SNAIL in renal cortex was detected using Western blot. Data are expressed as mean $\pm$ s.e.m. ($n = 6$). * $P < 0.05$ vs db/m; # $P < 0.05$ vs db/db.

SRT1720 inhibited HG-induced HIF1A, GLUT1 and SNAIL expressions in HK-2 cells

HIF1A expression was increased in HG-treated HK-2 cells, but this effect was reversed by SRT1720 treatment (Fig. 6A and C), suggesting that SIRT1 inhibited HIF1A expression in HG-treated HK-2 cells under normoxic conditions. Next, effects of SRT1720 on GLUT1 and SNAIL expressions were evaluated in HK-2 cells. Compared with GLUT1 and SNAIL expressions in the control group, expressions in HG group significantly increased, but SRT1720 treatment suppressed HG-induced increase in GLUT1 and SNAIL expressions

in HK-2 cells (Fig. 6). These data demonstrated that SRT1720 can suppress HIF1A, GLUT1 and SNAIL expressions in HG-induced HK-2 cells.

Knockdown of HIF1A or GLUT1 inhibited HG-induced EMT and ECM protein expression in HK-2 cells

To explore effects of HIF1A and GLUT1 on HG-induced EMT and ECM protein expression, HK-2 cells were transfected with siRNA against either *HIF1A* or *Glut1*. As shown in Fig. 7A, *HIF1A* knockdown efficiently reduced GLUT1 protein level in HG-induced HK-2 cells, but *GLUT1* siRNA

Figure 4

Effect of SRT1720 on expression of TGFβ1, CTGF, NF-κB p65, NOX4 and 8-OHdG in diabetic kidneys. (A) Immunohistochemical staining of TGFβ1, CTGF, NF-κB p65, NOX4 and 8-OHdG in kidney sections (Scale bar, 20 μm). (B) Protein levels of TGFβ1 and CTGF were assessed using Western blot. (C) NF-κB p65 expression in nuclear extracts was assessed using Western blot, and the relative intensity of nuclear NF-κB p65 was normalized against that of Histone H3. (D) NOX4 expression was detected using Western blot. (E) Twenty-four-hour urinary 8-OHdG excretion was measured using ELISA. Data are expressed as mean ± s.e.m. ($n = 6$). * $P < 0.05$ vs db/m; # $P < 0.05$ vs db/db.

did not affect HG-induced HIF1A expression. However, HG-induced upregulation of SNAIL was significantly declined in HK-2 cells transfected with *HIF1A* or *GLUT1* siRNA. Silencing *HIF1A* or *GLUT1* reversed effects of HG on E-cadherin and α-SMA expressions in HK-2 cells (Fig. 7B). Moreover, HG-induced fibronectin and collagen IV expressions were abolished by *HIF1A* or *GLUT1* siRNA in HK-2 cells (Fig. 7C). Overall, these results indicated that HIF1A and its target factors mediate HG-induced EMT and ECM protein expression in HK-2 cells.

Expressions of SIRT1 and HIF1A in renal tissue samples from patients with DN

SIRT1 and HIF1A expressions were assessed in renal tissue samples of patients with DN. Immunohistochemical staining showed that SIRT1 expression was markedly reduced in diabetic kidneys compared with that in normal kidneys (Fig. 8). Moreover, faint HIF1A staining was observed in normal kidneys, whereas prominent HIF1A staining was observed in diabetic kidneys (Fig. 8).

Figure 5

Effects of SRT1720 on HG-induced EMT, fibronectin and collagen IV expressions, and ROS generation in HK-2 cells. (A) Representative images of immunofluorescence staining for SIRT1, E-cadherin and α -SMA in HK-2 cells. (B) Expressions of SIRT1, E-cadherin and α -SMA were detected using Western blot. (C) SIRT1 activity in HK-2 cells. (D) Morphological changes of HK-2 cells were analysed under an inverted microscope. (E) Representative images of immunofluorescence for fibronectin and collagen IV in HK-2 cells. (F) Fibronectin and collagen IV expressions were detected using Western blot. (G) Mitochondrial ROS generation was assessed using the fluorescence probe MitoSOX Red. Note: Ctrl, 5.6 mM glucose plus 24.4 mM mannitol; HG, high glucose (30 mM); HG + SRT, HG + SRT1720 (2.5 μ M). Data are expressed as mean $\pm$ S.E.M. ($n = 6$). * $P < 0.05$ vs Ctrl; # $P < 0.05$ vs HG.

Figure 6

Effect of SRT1720 on expression of HIF1A, GLUT1 and SNAIL in HK-2 cells. (A) Representative images of immunofluorescence staining for HIF1A and GLUT1. NC, immunofluorescence images obtained from three group without primary antibody as a negative control. (B) Representative images of immunocytochemistry for SNAIL. NC, immunocytochemistry images obtained from three group without primary antibody as a negative control. (C) Expressions of HIF1A, GLUT1 and SNAIL were assessed by Western blot. Note: Ctrl, 5.6 mM glucose plus 24.4 mM mannitol; HG, high glucose (30 mM); HG + SRT, HG + SRT1720 (2.5 μM). Data are presented as mean ± S.E.M. ($n = 6$). * $P < 0.05$ vs Ctrl; # $P < 0.05$ vs HG.

Figure 7

Effects of *HIF1A* or *GLUT1* knockdown on expression of SNAIL, E-cadherin, α-SMA, fibronectin and collagen IV in HG-treated HK-2 cells. HK-2 cells were transfected with *HIF1A* siRNA or *GLUT1* siRNA and treated with HG for 48 h. (A) Protein levels of HIF1A, GLUT1 and SNAIL were detected using Western blot. (B) Protein levels of E-cadherin and α-SMA were detected using Western blot. (C) Protein levels of fibronectin and collagen IV were detected using Western blot. Note: HG, high glucose (30 mM); HG + Scr, HG + scramble; HG + siHIF1A, HG + *HIF1A* siRNA; HG + siGLUT1, HG + *GLUT1* siRNA. Data are expressed as mean ± S.E.M. ($n = 6$). * $P < 0.05$ vs HG.

Figure 8

Expressions of SIRT1 and HIF1A in kidneys of patients with DN or normal control kidneys were detected using immunohistochemistry staining. Arrows indicate the positive SIRT1 or HIF1A staining. Scale bar, 50 μ m. Negative control, immunohistochemistry images obtained from four groups without primary antibody as a negative control.

These observations were consistent with those in animal models, further supporting the notion that abnormal SIRT1 and HIF1A expression were associated with DN development.

Discussion

This study was undertaken to assess whether SRT1720, an SIRT1 activator, can ameliorate renal dysfunction and attenuate renal fibrosis in DN and to elucidate the possible underlying mechanisms. Our results show that SIRT1 expression and activity were decreased in kidneys of db/db mice and administration of SRT1720 reversed these changes. SRT1720 significantly attenuated glomerular

hypertrophy, mesangial expansion, glomerulosclerosis and interstitial fibrosis in db/db mice. In addition, *in vitro* results demonstrated that SRT1720 inhibits HG-induced fibrotic responses through SIRT1/HIF1A/GLUT1/SNAIL pathway.

Numerous studies have shown that SIRT1 exerts protective effects against DN by reducing oxidative stress, apoptosis, inflammation and lipotoxicity (Kitada *et al.* 2011, Kim *et al.* 2013). In this study, decreased SIRT1 expression was detected in kidneys of db/db mice, as well as in samples of patients with DN. This result is consistent with previous reports (Kim *et al.* 2013, Lo *et al.* 2017). Several studies identify NOX4 as a key source of ROS responsible for kidney injury in diabetes (Gorin *et al.* 2005, Jha *et al.* 2014). Inhibition of NOX4 alleviated

oxidative stress and renal injury in diabetic nephropathy (Jha *et al.* 2014). Previous studies have shown that NF- κ B activation has a role in renal inflammation and fibrosis of the progression of DN (Schmid *et al.* 2006, Navarro-Gonzalez *et al.* 2011). Reportedly, SIRT1 mediated anti-inflammatory and anti-oxidative effects by inhibiting NF- κ B activation and NOX4 expression, thereby protecting against kidney injury under diabetic conditions (Liu *et al.* 2014, Li *et al.* 2017a). Our findings demonstrated that SRT1720 administration inhibited NF- κ B activation, NOX4 expression and oxidative stress via SIRT1 induction in diabetic nephropathy.

Tubulointerstitial fibrosis is important for the prognosis of DN (Gilbert & Cooper 1999). Previous studies have confirmed that TGF β 1 play a central role in various fibrogenic processes, including ECM accumulation, and glomerular and tubulointerstitial fibrosis (Lan 2011). CTGF acts downstream of TGF β 1 to promote chronic tubulointerstitial fibrosis in DN (Wang *et al.* 2001). In our study, SRT1720 ameliorated ECM accumulation, renal fibrosis and TGF β 1 and CTGF expressions in the kidneys of db/db mice. These results suggest that SRT1720 inhibits renal fibrosis through inhibition of TGF β 1 and CTGF expression in diabetic mice.

HIF1A is highly expressed in glomerular and tubulointerstitial lesions of diabetic animal models and patients with DN (Higgins *et al.* 2007, Rosenberger *et al.* 2008, Isoe *et al.* 2010, Takiyama *et al.* 2011). YC-1, an HIF-1 inhibitor, reduced renal HIF1A expression and exerted promising renal protective effect in type 1 diabetic mice (Nayak *et al.* 2016). GLUT1 is constitutively expressed in diabetic kidney and promotes hyperglycemia-induced renal injury (Mogyorósi & Ziyadeh 1999, Ricci *et al.* 2006). Renal GLUT1 expression was significantly inhibited by YC-1 treatment in diabetic OVE26 mice (Nayak *et al.* 2016). Besides, GLUT1 was defined as a critical downstream target of HIF-1 α mediating high glucose-induced matrix expression through the NOX4 in glomerular mesangial cells (Nayak *et al.* 2016). SIRT1 has been reported to modulate HIF1A expression under hypoxic stress (Lim *et al.* 2010, Bae *et al.* 2013). In the present study, we observed SIRT1 activator SRT1720 inhibited HIF1A and GLUT1 expression in the kidney of db/db mice and HK-2 cells treated with high glucose. In addition, high glucose-induced GLUT1 expression was prevented by transfection of HIF1A siRNA in HK-2 cells. EMT is considered essential for the development of renal fibrosis in DN (Liu 2004). *Snail1* knockdown could reverse the fibrosis via inhibition of EMT in a fibrotic mouse model (Grande *et al.* 2015).

Moreover, HG-induced EMT was mediated through SNAIL in tubular epithelial cells (Lee & Han 2010, Zhao *et al.* 2017). These data indicate that SNAIL may be an effective target to block EMT in tubular epithelial cells and thereby may be protective against fibrosis. In the current study, we found that SRT1720 administration diminished EMT and SNAIL expressions *in vitro* and *in vivo*. Moreover, HIF1A or GLUT1 siRNA inhibited HG-induced EMT and SNAIL expression in HK-2 cells.

Previous studies have shown that lack of SIRT1 in β -cells leads to impaired glucose sensing and insulin secretion (Ramsey *et al.* 2008, Luu *et al.* 2013). SRT3025, an SIRT1 activator, ameliorated hyperglycemia, augmented plasma insulin concentrations, increased pancreatic islet beta cell mass and elevated hepatic beta cell growth factor beta-trophin expression in db/db mice (Gilbert *et al.* 2015). In Zucker fa/fa rats, SRT1720 improved glucose homeostasis and insulin sensitivity (Milne *et al.* 2007). In our study, SRT1720 reduced the blood glucose levels in db/db mice. Therefore, SRT1720 is likely involved in glucose homeostasis via SIRT1 induction in diabetic mice, suggesting that SRT1720 prevents diabetes-associated kidney injury partly through reduction of blood glucose levels.

In conclusion, the results from our *in vivo* and *in vitro* studies demonstrate that SRT1720 plays a protective role against DN by suppressing renal fibrosis. Moreover, our findings support the notion that SRT1720 reduces renal fibrosis via SIRT1/HIF1A/GLUT1/SNAIL pathway. Therefore, we speculate that SIRT1 can be a key target during DN treatment.

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.

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Author contribution statement

W H, Y S and H D contributed to the design, analysis and interpretation of data. Z Y, W H, L M, M W, N C and C D performed the study. C W performed tissue sectioning, staining, data acquisition and data analysis. W H, Z Y and Y S analysed the data, wrote the manuscript and revised the manuscript.

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