SIK2 protects against renal tubular injury and the progression of diabetic kidney disease

Bingyao Liu 1, Linlin Zhang 1, Hang Yang 1, Xinyu Chen 2, Hongting Zheng 1, Xiaoyu Liao 1,*

1 Department of Endocrinology, Chongqing Education Commission Key Laboratory of Diabetic Translational Research, the Second Affiliated Hospital of Army Medical University, Chongqing, China
2 Department of Pathology, Chongqing University Cancer Hospital, Chongqing, China

A B S T R A C T

Despite optimal medical therapy, many patients with diabetic kidney disease (DKD) progress to end-stage renal disease. The identification of new biomarkers and drug targets for DKD is required for the development of more effective therapies. The apoptosis of renal tubular epithelial cells is a key feature of the pathogenicity associated with DKD. SIK2, a salt-inducible kinase, regulates important biological processes, such as energy metabolism, cell cycle progression and cellular apoptosis. In our current study, a notable decrease in the expression of SIK2 was detected in the renal tubules of DKD patients and murine models. Functional experiments demonstrated that deficiency or inactivity of SIK2 aggravates tubular injury and interstitial fibrosis in diabetic mice. Based on transcriptome sequencing, molecular mechanism exploration revealed that SIK2 overexpression reduces endoplasmic reticulum (ER) stress-mediated tubular epithelial apoptosis by inhibiting the histone acetyltransferase activity of p300 to activate HSF1/Hsp70. Furthermore, the specific restoration of SIK2 in tubules blunts tubular and interstitial impairments in diabetic and vancomycin-induced kidney disease mice. Together, these findings indicate that SIK2 protects against renal tubular injury and the progression of kidney disease, and make a compelling case for targeting SIK2 for therapy in DKD.

Introduction

Diabetic kidney disease (DKD) is the leading cause of chronic kidney disease accounting for nearly 50% of all end-stage renal disease worldwide.1 Studies within the past years have demonstrated that renal tubular injury, and tubulointerstitial fibrosis and inflammation play important roles in DKD progression.2-4 Tubular atrophy is often characterized by the histopathological staining of kidney lesions in patients with DKD.5,6 Tubular epithelial cells are the primary targets of a variety of kidney injuries regardless of the initial insults. Injured tubular cells present the consequence phenomenon of cell proliferation, apoptosis, autophagy, and endothelial-mesenchymal transition.7,8 Accumulating evidence now indicates that the apoptosis of tubular epithelium is a crucial step in the pathogenesis of a variety of progressive kidney diseases, including DKD.9,10 Increased apoptosis has been observed in the proximal and distal tubular epithelium in patients with diabetes,11 as well as in tubular epithelial cells under high-glucose (HG) conditions.12 Thus, it is believed that the events leading to apoptosis in tubular epithelial cells and further progression to tubulointerstitial lesions are among the main features in DKD.13

Current therapies that aim to lower blood glucose have no effect on blocking renal damage, and co-treatment with renoprotective drugs often remains in toxicity, limiting the treatment efficacy. The underlying mechanisms predisposing to development and progression of DKD are an area of active investigation. Fundamental studies have implicated a number of cytokines, hormones, and intracellular signaling pathways, involved in oxidative stress, endoplasmic reticulum (ER) stress and mitochondrial function, in either the development or progression of DKD.14-16 ER stress, which is caused by the presence of unfolded or misfolded proteins, has both protective and deleterious features, depending on whether it is an initial adaptive response or prolonged activated...
At a Glance Commentary

Liao B, et al.

Background

Accumulating evidence indicates that the apoptosis of renal tubular epithelium is a crucial step in the pathogenesis of diabetic kidney disease (DKD).

Translational Significance

This study identified a notable decrease in the expression of SIK2 in the renal tubules of DKD patients and murine models. We demonstrated that deficiency of SIK2 increases renal tubular epithelial apoptosis, and aggravates tubular injury and interstitial fibrosis in DKD mice. Specific restoration of SIK2 in tubules blunts these impairments. Further mechanism exploration revealed that overexpression of SIK2 inhibits endoplasmic reticulum stress-induced tubular epithelial apoptosis by activating HSF1/Hsp70. These results suggested that restoring the expression of SIK2 in renal tubules might be an effective route for the treatment of DKD.

status. ER stress-induced cellular apoptosis contributes to tubular damages in kidney disease. An improved understanding of the molecules involved in regulating ER stress will help identify novel therapeutic strategies to prevent the progression of DKD.

Salt-inducible kinase 2 (SIK2) is a multifunctional kinase of the AMP-activated protein kinase (AMPK) family, members of which play crucial roles in sensing the energy state and stress response. Existing studies have revealed that SIK2 participates in energy metabolism and the modulation of cellular gene expression in response to hormones and nutrients. The knockdown of SIK2 downregulates GLUT4 expression and high-molecular-weight adiponectin levels in plasma, leading to reduced glucose uptake in muscle and white adipocytes. Specifically, SIK2 modulated the efficiency of insulin signal transduction by phosphorylating insulin receptor substrate 1 (IRS-1) on Ser794, and it also functions as a key gluconeogenic suppressor in the liver when combined with liver kinase B1 (LKB1). Additionally, SIK2 could inhibit p300 histone acetyltransferase (HAT) activity by direct phosphorylation at Ser89, resulting in a decrease in carbohydrate-responsive element–binding protein (ChREBP)-mediated lipogenesis in hepatocytes. Moreover, SIK2 also regulates the cell cycle and ER stress response via CREB-dependent transcription. These reports indicate that SIK2 has important effects in metabolic diseases such as diabetes. However, the role and functional mechanisms of SIK2 in DKD remains unexplored.

In this study, we found that both mRNA and protein expression levels of SIK2 in renal tubules of DKD patients and mouse models are obviously decreased. Functionally, we demonstrated that the decreased expression and repressed activation of SIK2 induces graver tubular epithelial apoptosis, and exacerbates tubular injury in DKD mice. Importantly, the restoration of SIK2 expression specifically in tubules attenuates tubular injury and interstitial fibrosis, which might be the results of the inhibition of ER stress-mediated tubular epithelial apoptosis through activating HSF1/Hsp70. Taken together, our results highlight the potential of SIK2 as a future therapeutic option for the treatment of DKD.

Methods

Human kidney specimens

Kidney biopsy tissues from the participants with DKD (n = 3) and without DKD (n = 3) were recruited for the study. The clinical characteristics of the participants were listed in Supplementary Table 1. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Army Medical University, Chongqing, China (clinical trial register no. ChiCTR-ROC-17010719), and informed written consent was obtained from all participants. The methods were conducted in accordance with the relevant guidelines and regulations. Kidney tissues were fixed in formalin, embedded in paraffin, and sectioned to 4 μm thickness. The tissue sections were stained with periodic acid Schiff (PAS, Solarbio) and Masson’s trichrome to assess kidney histological features.

Mice and treatment

db/db (BKS-Lepr−/−), littermate control for db/db (BKS-Lepr+/+), SIK2 knockout (SIK2 KO, B6J-SIK2−/−) and SIK2 wild-type (SIK2 WT, B6J-SIK2+/+) male mice were purchased from GemPharmatech Co., Ltd., China. All mice were housed under a 12 hour light/dark cycle at 25°C, and accessed water and food freely. SIK2 KO mice were generated with CRISPR-Cas9 technology. According to the structure of SIK2 gene, exon2 - exon3 of SIK2-201 (ENSMUST000004137510). transcript is recommended as the knockout region. The region contains 181 bp coding sequence. Knock out the region will result in disruption of protein function. Type 1 diabetic mice were induced as previously described, and were euthanized after 16 weeks. Briefly, C57BL/6, SIK2 WT and SIK2 KO mice received streptozotocin (STZ, Sigma-Aldrich) (50 mg/kg, pH = 4.5) through intraperitoneal (i.p) injection for 5 consecutive days. At 2 weeks post-injection, fasting glucose levels (6-h fast) were measured, and mice with a fasting glucose level above 13.8 mM were considered diabetic and used for this study. For acute tubular injury, C57BL/6 mice were i.p. injected with vancomycin (MedChemExpress) at a dose of 600 mg/kg for 6 days.

To inhibit SIK2 activity in STZ-induced diabetic mice, ARN-3236 (MedChemExpress) was administered p.o. at a dose of 60 mg/kg daily for 21 days. To evaluate the roles of SIK2 in tubular injury, STZ-induced diabetic and db/db mice were transduced with 2 × 1011 v.g. of AAV9-pCDH16-SIK2 or control vehicle AAV9-pCDH16 (Shanghai Genechem Co., Ltd.) via tail vein injection. To confirm the molecular mechanism of SIK2 in improving tubular injury, diabetic SIK2 KO and WT mice were transduced with 2 × 1011 v.g. of AAV9-pCDH16-HSF1 or control vehicle AAV9-pCDH16 (Shanghai Genechem Co., Ltd.) via tail vein injection. The STZ-induced diabetic mice were treated with ARN-3236 or AAV after 10–12 weeks of diabetic model identification. The mice were euthanized 4 weeks after AAV infection, and blood and kidneys were collected for analysis. The details of reagents were listed in Supplementary Table 2.

All animal studies were conducted according to protocols approved by the Laboratory Animal Welfare and Ethics Committee of the Army Medical University (AMUWEC20201508).

Urinary albumin, β-NAG, γ-TG and blood urea nitrogen assessment

Urine albumin, creatinine, β-NAG, γ-TG and blood urea nitrogen (BUN) were detected by commercial assay kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer’s protocol. Urine albumin to creatinine ratio (UACR) was calculated by urine albumin/urine creatinine. The details of commercial assay kits were listed in Supplementary Table 2.

Hematoxylin and eosin (H&E), Masson’s trichrome, PAS and immunohistochemical (IHC) staining

H&E, Masson’s trichrome, PAS, and IHC staining were performed as previously described. In brief, kidney samples were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Tissue sections were stained with commercial H&E, Masson’s trichrome and PAS staining kits (Solarbio). The interstitial fibrosis score was evaluated by examining 9 fields in randomly selected tissue samples stained with Masson’s trichrome. Blue-stained scarred areas were quantified by ImageJ. The interstitial fibrosis scores were expressed as the percentage of the scanned blue interstitium. Tubular injury was evaluated with ten randomly selected fields from H&E-stained sections, based on the histopathological changes of tubules, that is tubular atrophy, lumen dilatation, epithelial cellular detachment or vacuolar degeneration, and
luminal cast formation. Tubular injury was scored semiquantitatively on a scale of 0 to 4 based on the percentage of damaged tubules: 0, normal; 1, < 25%; 2, 25~50%; 3, 50~75%; and 4, > 75%.33,34

**Immunofluorescence (IF) staining and TUNEL assay**

IF staining was performed as described previously.35 Briefly, frozen kidney sections were incubated with the indicated primary antibodies overnight at 4°C, followed by incubation with the corresponding secondary antibodies. The following antibodies were used as primary antibodies: anti-AQP1 (Santa Cruz, 1:200) and anti-NGAL (Abcam, 1:100). TUNEL staining was conducted with an in situ cell death detection kit (Roche) according to the manufacturer’s instructions. All images were obtained using an Olympus fluorescence microscope.

**Transmission electron microscopy (TEM)**

Kidney cortex tissues (1 mm × 1 mm × 3 mm) were fixed in 2.5% glutaraldehyde and post fixed in osmic acid for 2 hours. The tissues were dehydrated in an ascending series of acetone, infiltrated with mixed solution (acetone: resin = 1:1) for 3 hours, and then embedded and polymerized for 24 hours. Resin-embedded blocks were sectioned to 70 nm and collected on 200-mesh, formvar-coated copper grids. Grids were stained with uranyl acetate and lead citrate, and examined using a JEM-1400Plus electron microscope.

**Cell culture**

HK2 cells were purchased from Cellcook Biotech (Guangzhou, China), and cultured in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12, Gibco) supplemented with 10% fetal bovine serum (FBS, Excell, China) and 1% penicillin-streptomycin antibiotics (Gibco). The cells were incubated at 37°C in a humidified atmosphere under 5% CO2. To induce ER stress, HK2 cells were stimulated with 1 μM thapsigargin (Sigma-Aldrich) for 24 hours.

**Plasmid construction, siRNA and plasmid transfection**

SIK2, p300 and HSF1 overexpression vectors were constructed by cloning the coding sequence into the pCMV3 vector. A SIK2 truncation mutant (SIK2_mut) was constructed with deletion of its kinase domain (20~271 aa). The constructed vectors were identified by Sanger sequencing. siRNA was purchased from RiboBio (Guangzhou, China). siRNA and plasmid were transfected with Lipofectamine RNAiMAX (Invitrogen) or Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions, respectively. The siRNA sequences are listed in Supplementary Table 3.

**RNA sequencing and data analysis**

Total RNA was extracted using TRizol reagent (Invitrogen). The RNA quality was determined by a 2100 Bioanalyzer (Agilent) and quantified using ND-2000 (Nanodrop Technologies). A transcriptome library was prepared using the TruSeqTM RNA sample preparation kit from Illumina (San Diego, CA) using 1 μg of total RNA. RNA sequencing was carried out with an Illumina HiSeq xten/Novaseq 6000 sequencer (2 × 150 bp read length). After quality control and filtration, clean reads were aligned to the reference genome with orientation mode using HISAT2 software (http://ccb.jhu.edu/software/htsat2/index.shtml).36 The mapped reads of each sample were assembled by StringTie (http://ccb.jhu.edu/software/stringtie/index.shtml? t = example) in a reference-based approach.37 Finally, the sequencing data were analyzed on the online platform I-Sanger of Majorbio.

**Apoptosis analysis**

Apoptosis was measured by flow cytometry using an Annexin V-PE/7-AAD apoptosis detection kit (BD Biosciences). HK2 cells were transfected with SIK2 overexpression vector or siRNA for 24 hours, and treated with 2 μM Tg for 24 hours. Then, the treated cells were harvested, washed with ice-cold phosphate-buffered saline (PBS) and resuspended. Then, the samples were incubated with Annexin V-PE and 7-AAD for 30 minutes at room temperature, resuspended in 400 μl binding buffer, and detected using FACScaliber Flow cytometer (Beckman).

**Short-Term Cell Proliferation Assays**

The transfected cells were placed into 96-well plates at 4 × 103 cells per well, and incubated for 5 days. The MTT (methylthiazooldiphenyl-tetrazolium bromide, Beyotime) assay was performed as previously described.38

**Reactive oxygen species (ROS) production assay**

ROS generation in kidneys was detected by MitoSOX red mitochondrial superoxide indicator (Invitrogen) as previously described.39,40 Briefly, cryostat sections from frozen tissues were incubated with MitoSoox reagent working solution (5 μM) for 10 minutes at 37°C. Then, the stained samples were washed with Hanks’ balanced salt solution (HBSS) buffer, and mounted in anti-fluorescence quenching sealing liquid. Stained images were obtained using an Olympus fluorescence microscope.

**Immunoblot assay**

Tissues or cells were lysed with a buffer containing protease inhibitor cocktail. After protein concentration determination, cell lysates were subjected to immunoblot analysis using specific antibodies (Supplementary Table 4).

**Immunoprecipitation (IP)**

HK2 cells were transfected with SIK2-HA, P300-flag, or HSF1-myc. Cells were lysed with lysis buffer and incubated with anti-Myc antibody or anti-Flag antibody overnight at 4°C, and the precipitated materials were used for immunoblot analysis using anti-acetylated-lysine or anti-HA antibody. For the detection of acetylated HSF1, HK2 cells were treated with MG132 (Santa Cruz Biotechnology) at 25 ng/ml for an additional 6 hours after overexpression plasmid transfection. The antibodies used in IP are listed in Supplementary Table 4.

**Dual-luciferase reporter gene assay**

A dual-luciferase reporter gene assay was performed as described previously.41 293T cells were co-transfected with overexpression plasmids HSF1-pCMV3, SIK2-pCMV3 or SIK2_mut-pCMV3, luciferase reporter HSPA6_promoter-firefly and TK-Renilla luciferase (internal control). Firefly and Renilla luciferase activities were measured using a dual-luciferase reporter gene assay system (Promega) on a SpectraMax i3x reader (Molecular Devices).

**Quantitative Real-Time PCR (qRT-PCR)**

The total RNA from tissues or cells was isolated using RNAiso Plus (Takara) according to the manufacturer’s instructions. The RNA quality and concentration were measured by a Nanodrop 2000 (Thermo Fisher Scientific). One microgram of total RNA was reversely transcribed into cDNA by the PrimeScript RT Reagent Kit with gDNA Eraser (Takara). qRT-PCR was performed using SYBR Green (Takara) on an Applied Biosystems 7500 Real-time PCR system. The relative expression level of mRNA was calculated with the following equation: relative expression = 2−ΔΔCT target
gene) − Ct (GAPDH)). GAPDH or ACTB is the reference gene for normalization. The primer sequences are listed in Supplementary Table 3.

Statistical analysis

Data are presented as the mean ± S.E.M. Student’s t-test was used to analyze the data between two groups, except for the comparison of tubular injury scores between two groups, which was analyzed with the Mann-Whitney test. A P value < 0.05 was defined as statistically significant.

Results

SIK2 expression is decreased in the renal tubules of DKD patients and murine models

In patients with DKD, morphological changes in both the glomerular and tubulointerstitial compartments, including matrix increases in the glomerular mesangial area and interstitial fibrosis, were highlighted by PAS and Masson’s trichrome staining in kidney samples compared with individuals without DKD (N-DKD) (Fig 1, A and Figure S1a). IHC staining
showed that SIK2 expression was predominantly localized to renal tubules, and the expression level was obviously decreased in the tubules of DKD patients compared with that in N-DKD individuals (Fig 1, B). To confirm the expression pattern of SIK2 in DKD, we also examined SIK2 expression in murine models of type 1 diabetes (STZ-induced diabetic mice) and type 2 diabetes (db/db mice). Histological staining and blood and urine detection results revealed renal function impairments in diabetic mice, including notable tubulointerstitial fibrosis (Fig 1, C and D), albuminuria and...

Fig 2. Deletion of SIK2 exacerbates tubular injury in diabetic mice. SIK2 KO and WT mice were injected with STZ at a dose of 50 mg/kg daily for 5 days or with vehicle as a control. (A, B) Body weight and UACR levels of WT and KO mice with or without STZ. (C) H&E and Masson’s trichrome staining of kidney sections, and the semiquantification of tubular injury and interstitial fibrosis in each group of mice. (D) IF staining of AQP1 and NGAL in kidney tissue sections. (E) Detection of tubular epithelial apoptosis was performed with a TUNEL assay using an in situ cell death detection kit, and the quantification of TUNEL-positive cells in WT and KO mice with STZ. (F) TEM analysis of tubular mitochondrial morphology (asterisks, angulation of mitochondria or disintegration of cristae), and the quantification of mitochondrial aspect ratio in the indicated mice. (G) ROS generation in each group of mice was detected with MitoSox staining. Scale bars: c-e and g, 20 μm; f, 0.5 μm. n = 5 mice per group. The data in the bar blot are presented as the mean ± S.E.M., compared with WT-STZ: * P < 0.05, ** P < 0.01.
tubular dysfunction (Figure S1b-n). With IHC, qRT-PCR and immunoblot analysis, we confirmed that SIK2 was mainly located in tubules (Fig 1, C and D), and the expression levels were significantly lower in the renal tubules of diabetic mice than in the corresponding controls, with an inverse correlation with the induction of tubular injury-associated markers (NGAL, IGFBP7, TIMP2 and KIM1) (Fig 1, E−H). Taken together, the deficiency of SIK2 in renal tubules of DKD patients and mice indicates its potential role in tubular function and DKD progression.

Deletion of SIK2 exacerbates tubular injury in diabetic mice

To determine the functional role of SIK2 in renal tubules in vivo, we used SIK2 KO mice generated with CRISPR-Cas9 technology. The knockout of SIK2 was verified by IHC staining and immunoblot analysis of kidney tissues (Figure S2a). Compared with wild-type (WT) mice, KO mice had no difference in body weight, fasting blood glucose (FBG), or renal morphology and function under nondiabetic conditions. STZ-induced diabetic KO mice (KO-STZ) displayed a decrease in body weight, and an increase in UACR and BUN compared with diabetic WT mice (WT-STZ) (Fig 2, A and B and Figure S2c), although there was no difference in the FBG between the 2 groups (Figure S2b). Histological analysis showed tubular atrophy and an expansion of the tubulointerstitial compartment in kidneys of diabetic WT mice, which were aggravated in diabetic KO mice (Fig 2, C). Likewise, the staining of kidney sections with AQP1 (a marker of renal tubules) and NGAL (a marker of tubular injury) demonstrated greater tubular injury in KO mice compared
with WT mice under diabetic conditions (Fig 2, D). Moreover, more serious tubular damage was also reflected by a significant increase in the urinary excretion of β-N-acetyl-glucosaminidase (β-NAG) (Figure S2d) and the mRNA expression levels of tubular injury markers (NGAL, TIMP2 and IGFBP7) (Figure S2e), and an obvious increase in the tubular epithelial apoptosis in diabetic KO mice (Fig 2, E and Figure S2f).

In kidneys, because of the high energy demand in the mitochondrial-enriched tubular compartment, it is likely that mitochondrial dysfunction in the diabetic state could contribute to epithelial cell apoptosis, proteinuria, and the loss of renal functions. In TEM images, the deformation of mitochondria was mainly swollen in diabetic WT mice. In diabetic KO mice, the deformation was aggravated, reflected by angulation of mitochondria and disintegration of cristae (Fig 2, F). Mitochondrial dysfunction was confirmed by increased ROS generation (Fig 2, G). In addition, compared with the control group, diabetic KO mice showed graver fibrosis and inflammatory response in the renal cortex (Figure S2g, h). Collectively,

Fig 4. SIK2 represses apoptosis of tubular epithelial cells by inhibiting ER stress. (A–B) HK2 cells were transfected with SIK2 overexpression or control vector for 24 hours, and RNA was extracted to perform RNA sequencing. (A) Heatmap showing the top 20 upregulated and top 20 downregulated genes in SIK2-overexpressing HK2 cells. (B) GO analysis of differently expressed genes and the 10 highest-ranking biological process terms are shown. (C–F) HK2 cells were transfected with SIK2 overexpression vector or siRNA for 24 hours, and then treated with Tg (1–2 μM) for 24 hours. (C, D) protein and mRNA expression levels of genes involved in ER stress were detected by immunoblot and qPCR. (E) Immunoblot analysis of apoptosis-associated proteins, including cleaved Caspase 3 and Bcl-2. (F) Flow cytometric analysis of treated cells for apoptosis after double staining with Annexin V-PE and 7-AAD. (G, H) HK2 cells were transfected with siRNA and treated with TUDCA (100μM). Protein levels of SIK2, phosphorylated eIF2α and CHOP were detected by immunoblot (G). Apoptotic cells were detected by flow cytometric analysis (H). The data in the bar blot are presented as the mean ± S.E.M., *P < 0.05, **P < 0.01, ***P < 0.001, ns, no significance.
these results suggest that genetic deletion of SIK2 sensitizes mice to tubular dysfunction and DKD development under diabetic conditions.

**Inhibiting the kinase activity of SIK2 exacerbates tubular injury in diabetic mice**

To confirm the role of SIK2 deficiency in regulating tubular function, we also exposed STZ-induced diabetic mice to ARN3236, an orally active and selective inhibitor of SIK2. As shown in Fig 3, A, although both the mRNA and total protein levels of SIK2 had no significant changes, the phosphorylation of SIK2 at Thr175 in kidneys was obviously reduced after ARN3236 treatment, indicating the inhibition of the kinase activity of SIK2. Similar to the results observed in KO mice, ARN3236 treatment had no significant effect on FBG levels and body weight (Figure S3a, b), but obviously increased the levels of UACR and BUN in diabetic mice (Fig 3, B and Figure S3c). Compared to the control group, inhibition of SIK2 activity aggravated tubular damage and...
interstitial fibrosis in diabetic mice (Fig 3, C). Graver tubular damage was confirmed by the increased content of urinary β-NAG (Fig 3, D), the increased expression levels of tubular injury markers (Fig 3, E and Figure S3d), and the reduction of AQP1 (Fig 3, F) in the injured kidneys of ARN3236-treated mice. Moreover, ARN3236 treatment increased the apoptosis of tubular epithelial cells (Fig 3, G and H). In addition, among the components of renal tubules in ARN3236-treated mice, mitochondrial deformation and ROS generation were also exacerbated (Fig 3, I and J). These results indicate that the inhibition of SIK2 activity worsens the tubular epithelial cell apoptosis and tubular dysfunction in diabetic mice, and suggest that SIK2 might be involved in the modulation of tubular function through its kinase activity.

SIK2 represses tubular epithelial apoptosis by inhibiting ER stress

We next explored the molecular mechanism underlying the role of SIK2 in tubular function by RNA sequencing (RNA-Seq) analysis of HK2 cells transfected SIK2 overexpression or control vectors. Hierarchical clustering of the top 20 upregulated and top 20 downregulated genes showed distinct expression patterns between SIK2-overexpressing and control cells (Fig 4, A). Gene Ontology (GO) analysis showed that the biological processes affected by SIK2 included the cellular response to X.
Fig 7. Tubular-specific overexpression of SIK2 attenuates tubular injury in diabetic mice. (A–I) STZ-induced diabetic mice were infected with AAV9-pCDH16-SIK2 (STZ-SIK2) or control vehicle (STZ-C). (A) Immunoblot analysis of SIK2 protein levels in kidney tissues from STZ-SIK2 and control mice. (B–E) FBG, UACR, BUN and \( \beta \)-NAG levels in the indicated mice. (F) H&E and Masson’s trichrome staining of kidney tissue sections, and the semi quantification of tubular injury and interstitial fibrosis.

(J–Q) db/db control mice were infected with AAV9-pCDH16-SIK2 (db/db-SIK2) or control vehicle (db/db-C). (J) Immunoblot analysis of SIK2 protein levels in kidney tissues from db/db-SIK2 and control mice. (K–M) FBG, UACR, BUN and \( \beta \)-NAG levels in the indicated mice. (N) H&E and Masson’s trichrome staining of kidney tissue sections, and the semi quantification of tubular injury and interstitial fibrosis.
stress, cellular response to stimulus, misfolded protein binding and so on (Fig 4, B). Similarly, Kyoto Encyclopedia of Genes and Genome (KEGG) pathway analysis showed that protein processing in ER was notably regulated by SIK2 overexpression (Figure S4).

The accumulation of misfolded or unfolded proteins in the ER will trigger ER stress-induced apoptosis by the unfolded protein response (UPR).44 ER stress has been defined to be linked to DKD, renal fibrosis, and acute kidney injury.16,45,46 Therefore, we speculated that SIK2 might be involved in the regulation of tubular function by modulating ER stress. In this case, we detected and confirmed that the proteins involved in ER stress, phosphorylated eIF2α and CHOP, were obviously downregulated in HK2 cells overexpressing SIK2, when treated with thapsigargin (Tg) (Fig 4, C), Tg, an inhibitor of Ca2+-transporting ATPase known to induce ER stress and trigger apoptosis in eukaryotic cells,47 had no effect on the expression of SIK2. In contrast, knockdown of SIK2 with small interfering RNAs (siRNAs) upregulated the expression of these proteins (Fig 4, C). By qPCR, overexpressing SIK2 significantly reduced the mRNA expression levels of XBP1s, ATF4 and CHOP in HK2 cells, when treated with Tg (Fig 4, D). Conversely, knockdown of SIK2 aggravated ER stress (Fig 4, D). Since prolonged ER stress will cause cellular apoptosis, we assessed the regulatory effect of SIK2 on the viability of tubular epithelial cells. As shown in Fig 4, E and F, overexpression of SIK2 repressed Caspase 3 activation, upregulated Bcl-2 expression, and ultimately blunt Tg-induced apoptosis in HK2 cells. In contrast, knockdown of SIK2 increased Tg-induced apoptosis. However, SIK2 did not affect the proliferation of HK2 cells (Figure S5). Additionally, treatment HK2 cells with tauroursodeoxycholic acid (TUDCA), a chemical chaperone, attenuated the increased protein levels of phosphorylated eIF2α and CHOP (Fig 4, G), and the cellular apoptosis (Fig 4, H) induced by knockdown of SIK2. Taken together, these data suggest that SIK2 reduces tubular epithelial apoptosis by inhibiting ER stress.

SIK2 inhibits ER stress-induced tubular epithelial apoptosis by activating HSF1/Hsp70

According to RNA-seq data, the biological process affected by SIK2 overexpression is mainly related to the stress response, and the expression of heat shock protein 70 (Hsp70), such as HSPA6, was changed in HK2 cells. The increased synthesis of Hsp70 is a hallmark of stressed cells and organisms, which functions as molecular chaperones to prevent protein misfolding and aggregation to maintain protein homeostasis.48 Hsp70 has also been reported to alleviate ER stress and prevent apoptosis.49,50 Consequently, we speculate that SIK2 might inhibit ER stress through the upregulation of Hsp70. As shown in Fig 5, A, overexpression of SIK2 promoted the expression of HSPA6 and HSPA4, while knockdown of SIK2 inhibited their expression in HK2 cells. Heat shock factor 1 (HSF1) is a prerequisite for the transactivation of HSP genes, the maintenance of cellular integrity during stress, and the development of thermotolerance.25 Accordingly, knockdown of HSF1 with siRNA attenuated the upregulation of HSPA6 and HSPA4, and blunt the inhibition of ER stress and apoptosis mediated by overexpressing SIK2 in HK2 cells (Fig 5, B–D).

As SIK2 is a Ser/Thr kinase, we detected the phosphorylation of HSF1 in HK2 cells. However, the phosphorylation level of HSF1 was not significantly changed when SIK2 was overexpressed or knocked down (Fig 5, E). A previous study reported that HSF1 could be acetylated and inactivated by p300, an endogenous HAT.51 Intriguingly, the HAT activity of p300 can be repressed by SIK2 through direct phosphorylation at Ser89 of p300.25 In HK2 cells, with IP assay, we found that SIK2 interacted with p300 (Fig 5, F). As expected, overexpressing SIK2 promoted the phosphorylation of p300 (Ser89), and knockdown of SIK2 repressed the phosphorylation of p300 (Fig 5, G). Ultimately, the acetylation of HSF1 was inhibited by SIK2 overexpressing (Fig 5, H), which suggested that the activity of HSF1 was promoted. Additionally, in a dual-luciferase reporter gene assay, SIK2 distinctly promoted the HSF1-mediated transcriptional activation of HSPA6 in a dose-dependent manner (Fig 5, I), but the promotion was abated by truncated mutation of the kinase domain52,53 of SIK2 (Fig 5, J). Collectively, these results indicate that SIK2 inhibits ER stress-induced tubular epithelial apoptosis by, at least partly, repressing HAT activity of p300 to activate HSF1/Hsp70.

Tubular-specific overexpression of HSF1 reverses SIK2 deficiency-induced tubular injury in diabetic mice

Then, we further confirmed the results that HSF1 is involved in the mechanism by which SIK2 inhibits ER stress-induced apoptosis of epithelial cells in vivo. STZ-induced diabetic WT and KO mice were injected in the tail vein with a recombinant adeno-associated virus 9 (AAV9) vector containing the promoter of CDH16, a tubular-specific marker,54,55 and the coding sequence of HSF1 (i.e., AAV9-pCDH16-HSF1), which is a gene therapy approach to primarily transduce cells within the renal tubules.56–58 Overexpression of HSF1 in tubules was confirmed by IHC staining and immunoblot analysis (Fig 6, A). Compared with diabetic WT mice, diabetic KO mice had no difference in FBG levels, whether HSF1 was overexpressed or not (Fig 6, B). However, the decreased body weight, and the aggravated albuminuria, interstitial fibrosis, tubular injury and epithelial apoptosis observed in diabetic KO mice were attenuated by the tubular-specific overexpression of HSF1 (Fig 6, C–G). Meanwhile, in diabetic mice, overexpressing HSF1 abated the upregulation of genes involved in tubular injury and interstitial fibrosis induced by knockout of SIK2 (Fig 6, H and I). Moreover, the decreased HSPA6 and increased cleaved Caspase 3 levels in diabetic KO mice were blunted by overexpressing HSF1 (Fig 6, J), consistent with the molecular mechanism disclosed in vitro, which suggests that SIK2 deficiency accelerates the progression of tubular injury by inhibiting HSF1 activity.

Tubular-specific overexpression of SIK2 attenuates tubular injury in diabetic and vancomycin-induced mice

Finally, we verified the effects of rescuing SIK2 expression on reversing the phenotypes in multiple murine models of DKD. Tubular-specific overexpression of SIK2 was induced by injection of recombinant AAV9-pCDH16-SIK2 into the tail vein of diabetic mice, including STZ-induced and db/db mice. SIK2 overexpression in tubules was confirmed by immunoblotting and IHC staining of cortex tissues (Fig 7, A and J and Figure S6a, S7a). Similar to the observations in SIK2 KO mice, the expression level of SIK2 did not affect the FBG levels (Fig 7, B and K). However, restoration of SIK2 significantly reduced the albuminuria, BUN and β-NAG contents (Fig 7, C, E–I and O). In STZ-induced diabetic mice, the expression level of SIK2 inversely correlated with the renal function, with the exception of the albuminuria (Fig 7, C). Overall, SIK2 overexpression attenuated tubular injury in diabetic and vancomycin-induced mice.
Fig 8. Tubular-specific overexpression of SIK2 attenuates tubular injury in vancomycin-induced mice. C57BL/6 mice were infected with AAV9-pCDH16-SIK2 (VAN-SIK2) or control vehicle (VAN-C), 4 weeks later, the mice were i.p. injected with vancomycin at a dose of 600 mg/kg for 6 days. (A) IHC staining of SIK2 in kidney tissue sections from VAN-SIK2 and VAN-C groups. (B, C) Body weight and UACR levels in the indicated mice. (D) H&E and Masson's trichrome staining of kidney tissue sections, and the semi-quantification of tubular injury and interstitial fibrosis in each group of mice. (E) IF staining of AQP1 and NGAL in kidney tissue sections. (F) mRNA expression levels of genes involved in tubular injury in the indicated mice. (G) TUNEL assay to detect apoptotic tubular epithelial cells, and the TUNEL positive cells were quantified. (H) TEM analysis of tubular mitochondrial morphology and the quantification of mitochondrial aspect ratio. Asterisks, angulation of mitochondria or disintegration of cristae. (I) Mitosox staining to detect ROS generation in the cortex. (J, K) mRNA expression levels of genes involved in tubulointerstitial fibrosis (J) and the inflammatory response (K) in the indicated mice. (L) Immunoblot analysis of SIK2, HSPA6, ATF4, CHOP and cleaved Caspase 3 expression levels. Scale bars: A, D, E, G and I, 20 μm; h, 0.5 μm. n = 5 mice per group. The data in the bar blot are presented as the mean ± S.E.M., * P < 0.05, ** P < 0.01, *** P < 0.001, ns, no significance.
Figure S6b, S7b), and the reduction of tubular epithelial apoptosis in diabetic mice (Fig 7, Q and H, upper panel). Meanwhile, the diabetic mice overexpressing SIK2 showed an obvious improvement in mitochondrial morphology and function, with clearly elongated and less angulated mitochondria (Fig 7, H and Q, lower panel) and decreased ROS production compared with the respective controls (Fig 7, I and R). In addition, the expression levels of genes associated fibrosis and inflammation were also significantly reduced in diabetic mice overexpressing SIK2 (Figure S6c, d and Figure S7c, d). Moreover, we found that the protein levels of HSPA6 were notably increased, and ATF4, CHOP and cleaved Caspase 3 were decreased (Fig 7, G and P), consistent with the molecular mechanism disclosed in HK2 cells. Taken together, these results indicate that targeting SIK2 might be a potential therapeutic strategy for tubular injury in DKD.

To confirm the role of SIK2 in protecting against tubular injury, we also performed investigation on vancomycin (VAN)-induced mice, a model of non-diabetic kidney disease that is characterized by proinflammatory oxidation, mitochondrial dysfunction, cellular apoptosis and tubular injury.6,20,25 As shown in Figure S8, VAN-induced mice exhibited distinct tubular dysfunction and inflammatory response (Figure S8a-f), and a significant decrease in the protein and mRNA expression levels of SIK2 compared with control mice (Figure S8g, h). As expected, the tubular overexpression of SIK2 in VAN-induced mice (VAN-SIK2) had no effect on body weight (Fig 8, A and B), but ameliorated albuminuria (Fig 8, C), tubular injury and interstitial fibrosis (Fig 8, D). The improved tubular injury in VAN-SIK2 mice was confirmed by the increased protein levels of AQPI, and the decreased expression levels of injury markers of SIK2 expression level and tubular injury was functionally validated by SIK2 ablation and tubular-specific overexpression in multiple mouse models of kidney disease.7,2,63 In the present study, we revealed an important role of SIK2 in protecting against tubular injury in DKD and in VAN-induced kidney disease.

In DKD patients, the downregulation of SIK2 was found predominantly in the renal tubules. Consistent with the expression pattern of SIK2 in humans, we confirmed that SIK2 expression was markedly decreased in the tubules of DKD mice. Furthermore, the correlation between SIK2 expression level and tubular injury was functionally validated by SIK2 ablation and tubular-specific overexpression in multiple mouse models of kidney disease. As results, the overexpression of SIK2 reversed the kidney impairments induced by diabetes, primarily alleviating tubular epithelial apoptosis, mitochondrial dysfunction and interstitial fibrosis, suggesting that SIK2 exerts a beneficial effect in protecting against tubular injury in DKD.

SIK2, an AMPK-related protein kinase with a well-established role in the regulation of metabolism,8,20,25 has been reported to regulate autophagy when proteasomal function is compromised,9,25 and contribute to ER-associated protein degradation in mammalian cells.66 In our study, based on the transcriptome sequencing of tubular epithelial cells overexpressing SIK2, we found that SIK2 reduced the expression of ER stress markers (phosphorylated eIF2α, XBP1s, ATF4 and CHOP), and inhibited the apoptosis of HK2 cell. PERK-eIF2α-CHOP is an important pathway involved in ER stress, leading to the activation of apoptosis, which supports a role for SIK2 in inhibiting ER stress-induced tubular epithelial apoptosis. It has been reported that high glucose could induce ER stress and the apoptosis of tubular cells.67 Accumulating evidence demonstrates that ER stress plays a major role in the development and progression of kidney diseases, such as DKD.68-70 Elevated urinary protein excretion in humans is known to be associated with ER stress and tubular injury.67,71 In the past decade, there has been considerable interest in developing compounds that regulate ER stress response to therapy associated diseases. Chemical chaperones improving the folding capacity of ER, such as the ER chaperone ORP150 and tau-rimine-conjugated unsaturated-cholesterol, have been disclosed to repress ER stress, and restore glucose tolerance and insulin sensitivity.72-74 It indicates that SIK2 might be a novel marker targeting ER stress to prevent tubular damage in DKD.

With molecular mechanism exploration, we also identified that SIK2 upregulates the expression of HSPA6 and HSPA4, members of Hsp70 family. Hsp70 has been shown to inhibit apoptosis by preventing cytochrome c/dATP-mediated caspase activation and the recruitment of caspases to the Apaf-1 apoptosis complex.75 In addition, Hsp70 also leads to increased levels of Bcl-2, an antiapoptotic protein that acts to block the mitochondrial release of cytochrome c.76 The expression of Hsp70 is mediated by the release of HSF1 in response to stressful stimuli.77 The activation of HSF1 involves several levels of regulation, including posttranslational modification with acetylation, hyperphosphorylation, and sumoylation.78 It has been demonstrated that the anti-diabetic agent exendin-4 promotes the SIRT1-mediated deacetylation of HSF1, which upregulates Hsp70 expression to alleviate ER stress.90,91 Interestingly, SIK2 was identified to inhibit the activity of a histone acetyltransferase, p300, by phosphorylation,72 and p300 has been reported to acetylate HSF1 to repress its activity.73 Likewise, in HK2 cells, we indeed found that overexpression of SIK2 promoted p300 phosphorylation to ablate its HAT activity, and reduced the acetylation of HSF1, consequently promoting its transactivation. Additionally, truncated mutation of the SIK2 kinase domain reduced the transactivation of HSF1 on HSPA6. Collectively, these results suggest that SIK2 activates HSF1/Hsp70 by inhibiting the HAT activity of p300, which might be involved in the mechanism of SIK2 inhibiting ER stress-induced cell apoptosis.

Mitochondria are enriched in renal tubules to meet high metabolic energy demand, and mitochondrial dysfunction can cause oxidative stress, cell apoptosis and tubular damage.80,81 In the tubules of DKD and VAN-induced mice, we also observed that tubular overexpression of SIK2 improved mitochondrial morphology and function with a decrease in ROS generation, and increased the expression levels of anti-oxidative stress markers (data not shown). Oxidative stress is a cytotoxic outcome of excessive generation of ROS, and can establish a progressive pathological cycle with ER stress.82,83 Prolonged ER stress and excessive ROS can cause cell apoptosis. ER stress induces Ca2+ release from the ER lumen to mitochondria, and increases ROS generation and the release of cytochrome C, which triggers the vicious cycle of oxidative stress both in the ER and mitochondria, eventually leading to cell apoptosis.84,85 In our study, overexpression of SIK2 attenuated ER stress, and improved mitochondrial function, ultimately reducing tubular epithelial apoptosis, which suggests that SIK2 might be involved in the regulation of ER stress-induced mitochondrial dysfunction and cellular apoptosis.

Our findings indicate that the deletion of SIK2 exacerbates tubular injury and tubulointerstitial fibrosis in DKD and VAN-induced kidney disease, and these events are reversed with the specific overexpression of SIK2 in tubules. The mechanism involves SIK2 directly depressing the HAT activity of p300 to activate HSF1, inhibiting ER stress and improving mitochondrial function, consequently reducing the apoptosis of tubular epithelial cells. This suggests that rescuing tubular SIK2 expression might have therapeutic potential for the treatment of DKD.

Data availability

The RNA sequencing data have been deposited in the NCBI SRA database under accession number SRA: SRP349039.
Author contributions

BY.L.: acquisition, analysis and interpretation of data, and statistical analysis; I.L.Z. and H.Y.: acquisition of data; XY.C.: statistical analysis; XY.L. and HT.Z.: study concept and design, revision of the manuscript, acquisition of funding.

Acknowledgments

This work was supported by grants from the National Science Fund for Distinguished Young Scholars of China (No. 81925007), the Natural Science Foundation of Chongqing (No. cstc2021jcyj-msxmX0465), the “Talent Project” of Army Medical University (No. 2019R055, 2019R003), and the Special Program for Basic Research Frontier of Military Medicine of the Second Affiliated Hospital of Third Military Medical University (No. 2018BYQY1006). The manuscript has been reviewed and approved by all named authors. All authors have read the journal’s authorship agreement, and declare that they have no potential conflicts of interest.

Supplementary materials

This article is associated with a supplementary article that can be found in the online version at doi:10.1016/j.trsl.2022.08.012.

Reference


