ORIGINAL RESEARCH COMMUNICATION

Cdk5-Mediated Phosphorylation of Sirt1 Contributes to Podocyte Mitochondrial Dysfunction in Diabetic Nephropathy

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Abstract

Aims: Mitochondrial dysfunction contributes to podocyte injury, which is the leading cause of proteinuria in diabetic nephropathy (DN). In this study, we explored the role of cyclin-dependent kinase 5 (Cdk5) in mitochondrial dysfunction of podocytes under diabetic conditions.

Results: Our results showed that the expression and activity of Cdk5 were significantly upregulated *in vivo* and *in vitro* under diabetic conditions, accompanied by the downregulation of synaptopodin and nephrin, as well as structural and functional mitochondrial dysfunction. Inhibition of Cdk5 with roscovitine or dominant-negative Cdk5 led to the attenuation of podocyte injury by upregulating synaptopodin and nephrin. The inhibition of Cdk5 also ameliorated mitochondrial dysfunction by decreasing reactive oxygen species levels and cytochrome c release, while increasing adenosine triphosphate production. Sirt1, an NAD⁺-dependent deacetylase, was decreased in podocytes with high glucose (HG) treatment; however, its phosphorylation level at S47 was significantly upregulated. We demonstrated that HG levels cause overactive Cdk5 to phosphorylate Sirt1 at S47. Suppression of Cdk5 reduced Sirt1 phosphorylation levels and mutation of S47 to nonphosphorable alanine (S47A), significantly attenuated podocyte injury and mitochondrial dysfunction in diabetic condition *in vivo* and *in vitro*.

Innovation and Conclusion: Our study has demonstrated the role of Cdk5 in regulating mitochondrial function through Sirt1 phosphorylation and thus can potentially be a new therapeutic target for DN treatment. IRB number: 20190040. *Antioxid. Redox Signal.* 34, 171–190.

Keywords: diabetic nephropathy, mitochondrial dysfunction, podocyte, Cdk5, Sirt1

Introduction

AS ONE OF THE MOST COMMON and serious microvascular complications of diabetes, diabetic nephropathy (DN) is one of the primary causes of end-stage renal disease worldwide (28, 38). Podocytes are terminally differentiated epithelial cells of the kidney, and their body extends interdigitated foot processes (FPs) forming the crucial component of the glomerular filtration barrier that prevents protein leakage (39). Injury, dedifferentiation, detachment, and apoptosis of podocytes are pathological hallmarks of DN, which promotes proteinuria and eventually lead to glomerulosclerosis (37, 45).

Mitochondria are key organelles of cellular bioenergetics and constantly undergo dynamic remodeling processes, including fusion and fission to adjust to cellular demand for energy (6). Podocytes cannot regenerate after differentiation and maturation, and their proliferative and regenerative abilities are extremely limited. Therefore, many mitochondria are needed in their cytoplasm to provide energy for maintaining cell structure and function. Emerging evidence suggests that mitochondrial dysfunction influences the development and progression of DN, especially during podocyte injury (41, 49). Mitochondrial dysfunction induced by high glucose (HG) level, with excess reactive oxygen species (ROS) production, is a major reason

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Innovation

Mitochondrial dysfunction contributes to podocyte injury, which is the leading cause of proteinuria in diabetic nephropathy (DN). The mechanism of mitochondrial dysfunction in podocytes in diabetes remains to be fully elucidated. Our investigation reveals a novel role for cyclin-dependent kinase 5 (Cdk5) in regulating mitochondrial function through Sirt1 phosphorylation. We found that Cdk5 contributions to podocyte mitochondria dysfunction are significantly dependent on its close interactions with Sirt1, thus phosphorylating Sirt1 *in vitro* and *in vivo*. Our findings unraveled prospects for the potential targeting of mitochondrial functions by modulating Cdk5 activities as a treatment strategy against DN.

for podocyte injury in diabetes (42, 58). The contribution and mechanism of mitochondrial dysfunction to DN onset or progression remain unclear, especially in podocyte injury. Studies have shown that ROS generation and oxidative stress caused by HG promote podocyte apoptosis (15, 42).

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine kinase that belongs to the family of cyclin-dependent kinases (Cdks) (8). Unlike other Cdk members, Cdk5 activity is primarily controlled by specific co-activators, p35 and p39 (24). The predominance of both Cdk5 activators and Cdk5 activity was initially discovered in neuronal cells and tissues (52). Cdk5 plays an important role in regulating central nervous system development and synaptic plasticity (10). However, deregulation of the Cdk5 activity has cytotoxic consequences in cells and is associated with mitochondrial dysfunction in neurons, a leading cause of the development of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (36).

Recently, studies have found that Cdk5 is also present in multiple nonneuronal cells and tissues (33). It was predominantly detected in podocytes from the capillary loop stage up to maturation and persisted in the podocytes of adult glomeruli; it plays important roles in podocyte differentiation, proliferation, and morphology (7, 57). It has been demonstrated that HG and transforming growth factor- β 1 (TGF- β 1) induced podocyte apoptosis by increasing the expression of Cdk5 (55). Whether Cdk5 participates in podocyte injury by inducing mitochondrial dysfunction in DN has not been clarified.

Sirt1, a silent information regulator, is a member of the deacetylase family and is involved in physiological and pathological processes such as cell senescence, metabolism, and apoptosis (2). In the past decade, Sirt1 has been found to promote the steady-state energy remodeling of kidney cells in high glucose environments, regulate oxidative stress, resist

renal cell apoptosis, inhibit kidney inflammation, and improve renal fibrosis during the onset of DN (1, 5, 23, 53). Thus, Cdk5 seems to be of special interest in the context of Sirt1 regulation. Studies have shown that Cdk5-mediated hyperphosphorylation of Sirt1 contributes to the development of endothelial senescence and atherosclerosis (4).

In this study, we investigated the direct interplay between Cdk5 and Sirt1 in podocyte injury under diabetic conditions. Importantly, we explored the novel role of Cdk5 in regulating mitochondrial function through phosphorylation of Sirt1, which could lead to immense opportunities to target mitochondrial function and Cdk5 in the treatment of DN.

Results

Cdk5 is increased in the glomeruli of patients with DN and diabetic mice

To validate the expression level of Cdk5 in the glomerulus, the renal biopsies from patients pathologically diagnosed with DN were analyzed by immunohistochemistry. In control tissues, low level of Cdk5 was seen in the glomeruli, whereas significantly stronger expression of Cdk5 was detected in the glomeruli of patients with DN (Fig. 1A, B). In contrast to Cdk5, nephrin and synaptopodin were markedly down-regulated in DN (Fig. 1C–F). Furthermore, statistical analysis demonstrated that the Cdk5 staining intensity negatively correlated with the intensity of nephrin and synaptopodin (Fig. 1G, H). Immunofluorescence double-labeling experiments indicated that Cdk5 colocalized with nestin, a marker of podocyte, in the glomerulus (Fig. 1I, J).

We next detected the expression of Cdk5 in diabetic mice. Compared with the control group, the immunohistochemical analysis showed that the expression levels of Cdk5 were significantly increased in streptozotocin (STZ)-induced diabetic mice and db/db mice, along with the decreased expressions of nephrin and synaptopodin (Fig. 1K–R).

HG upregulates the expression and activation of Cdk5 in human podocytes

To explore the effects of hyperglycemia on Cdk5 expression, human podocytes were treated with 30 mM glucose for 0, 12, 24, 48, and 72 h. As the markers of podocyte injury, the expression levels of nephrin and synaptopodin were significantly decreased by HG treatment (Fig. 2A–C). Upregulation of Cdk5 and TGF- β 1 was observed in podocytes after 12 h HG treatment, and the expression reached its highest level at 24 and 48 h (Fig. 2A, D, E). p25, cleaved from the Cdk5 activator p35, was also significantly upregulated after 12 and 24 h (Fig. 2A, F, G). Kinase activity analysis showed that the level of p-Histone H1 was significantly increased by HG treatment at 24 h, which demonstrated that HG conditions may lead to overactive Cdk5

FIG. 1. Cdk5 increased in the glomeruli of patients with DN and diabetic mice. (A, C, E) Representative immunohistochemical staining for Cdk5, synaptopodin, and nephrin in the glomerulus. (B, D, F) Quantification of Cdk5-, synaptopodin-, and nephrin-positive area in the glomerulus. ($p < 0.05$, $n = 19$) (G, H) The correlation between Cdk5 with synaptopodin and nephrin levels in DN patients was evaluated by the Spearman correlation analysis. (I, J) Colocalization of Cdk5 and nestin in the glomerulus, DAPI is a nucleus dye. (K, L) Representative immunohistochemical staining for Cdk5, synaptopodin, and nephrin in the glomerulus from diabetic mice. (M–R) Quantification of Cdk5-, synaptopodin-, and nephrin-positive area in the glomerulus. ($p < 0.05$). Scale bars: 50 μ m. Magnification: 400 $\times$. Cdk5, cyclin-dependent kinase 5; DN, diabetic nephropathy. Color images are available online.

FIG. 2. HG upregulated the expression and activation of Cdk5 in human podocytes. (A) Protein representative Cdk5, P35/25, TGF-β1, synaptopodin, and nephrin in HPCs induced by 30 mM glucose for different time points. (B–G) Protein levels of Cdk5, P35/25, TGF-β1, synaptopodin, and nephrin. (H) Protein representative photographs of Histone H1 and phospho-Histone H1 in HPCs induced by HG. (I) Protein levels of phospho-Histone H1. (J) Protein representative photographs of TGF-β1 and Cdk5 in HPCs treated by LY364947 in 24 h HG conditions. (K, L) Protein levels of TGF-β1 and Cdk5. The data represent the mean $\pm$ SD ($n = 3$). p-, phospho-. * $p < 0.05$ compared with the control group, # $p < 0.05$ compared with the HG + DMSO group. Please refer to Supplementary Figure S1 for raw Western blot records shown in the figure. HG, high glucose; HPC, human renal podocyte.

(Fig. 2H, I). Human renal podocytes (HPCs) were treated with LY364947, an inhibitor of TGF-β1, in 24 h HG conditions, which caused a decrease in Cdk5 expression (Fig. 2J–L). These results verified that hyperglycemia, possibly *via* TGF-β1, upregulates and activates Cdk5 (Supplementary Fig. S1).

Cdk5 suppression attenuates HG-induced podocyte injury by alleviating mitochondrial dysfunction

To confirm whether Cdk5 is directly associated with podocyte injury, roscovitine, a Cdk5 inhibitor, was used to suppress the Cdk5 kinase activity and expression. Roscovitine

cytotoxicity was detected by CCK8. The results showed that the viability of cells was $<40\%$ after treatment with $20 \mu\text{M}$ roscovitine (Fig. 3A). Thus, for subsequent experiments, we selected 2.5, 5, and $10 \mu\text{M}$ roscovitine to treat podocytes. As shown in Figure 3, treatment with roscovitine significantly recovered the expression of nephrin and synaptopodin, which decreased significantly after HG stimulation, as detected by Western blotting, quantitative real-time polymerase chain reaction (RT-qPCR), and immunofluorescence. These results demonstrated that the increased expression level of Cdk5 induced by HG may be associated with podocyte injury in diabetes (Fig. 3B–F, Supplementary Fig. S2).

FIG. 3. Roscovitine, the inhibitor of Cdk5, attenuated podocyte injury induced by HG at 24 h. (A) The cytotoxicity detection of roscovitine by CCK8. $*p < 0.05$ compared with the 0 μM group. (B) Protein representative Cdk5, P35/25, synaptopodin, and nephlin in HPCs. (C, D) Protein levels of Cdk5, P35/25, synaptopodin, and nephlin. (E) The mRNA levels of synaptopodin and nephlin were analyzed by RT-qPCR. (F) The expression of Cdk5, P35/25, synaptopodin, and nephlin by immunofluorescence. DAPI is a nucleus dye. Scale bar: 25 μm. The data represent the mean $\pm$ SD ($n = 3$). C, control; M, mannitol; R, roscovitine. $*p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the HG group. Please refer to Supplementary Figure S2 for raw Western blot records shown in the figure. RT-qPCR, quantitative real-time polymerase chain reaction. Color images are available online.

Increasing evidence indicates that the dysfunction of mitochondrial plays many roles in DN pathogenesis (25, 26). To assess the role of Cdk5 in podocyte mitochondrial dysfunction, we first detected the expression of dynamic-related protein 1 (Drp1) and mitofusin 1 (Mfn1). The up-regulation of Drp1, a mitochondrial fission protein, was observed in HG-treated podocytes, and especially, the

phosphorylation level of Drp1 increased significantly (Fig. 4A, B, Supplementary Fig. S2). However, Mfn1, a mitochondrial fusion protein, was significantly decreased in podocytes (Fig. 4A, B, Supplementary Fig. S2). Furthermore, we detected an upregulation in the expression of mitochondrial damage-related protein, Cyto C, in HG-treated podocytes at 24 h (Fig. 4A, B, Supplementary Fig. S2).

FIG. 4. Cdk5 suppression alleviated the HG-induced mitochondrial dysfunction at 24 h. (A) Protein representative Drp1, p-Drp1, Mfn1, SOD2, and CytoC in HPCs. (B) Protein levels of Drp1, p-Drp1, Mfn1, and CytoC. (C, D) The MitoTracker™ Deep Red FM (50 nM) was visualized under confocal microscopy and it was quantified. Scale bar: 10 μm. (E, F) The MitoSOX™ Red was visualized under confocal microscopy and it was quantified. Scale bar: 10 μm. (G, H) ROS generation was measured by flow cytometry and was quantified following DCFH-DA (10 μM) staining. (I, J) The mitochondrial membrane potential in HPCs was measured by JC-1 staining using confocal microscopy. Scale bar: 10 μm. (K, L) The mitochondrial membrane potential was measured by flow cytometry and was quantified following JC-1 (100 μM) staining. (M, N) Representative micrographs showing SA-β-gal staining in HPCs. Scale bar: 50 μm. (O) ATP concentrations were determined using an ATP determination kit. The data represent the mean $\pm$ SD ($n = 3$). C, control; M, mannitol; R, roscovitine; p-, phospho-. $*p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the HG group. Please refer to Supplementary Figure S2 for raw Western blot records shown in the figure. ROS, reactive oxygen species. Color images are available online.

The mitochondrial morphology in human podocyte was examined by MitoTracker Red staining. In physiological conditions, mitochondria appeared thread-like in human podocyte. Treatment with HG markedly affected mitochondrial shape; elongated mitochondria became much shorter and rounder in morphology under these conditions. In contrast, pretreatment with Cdk5 inhibitor, roscovitine, or knockdown of Cdk5 with DN-Cdk5, significantly prevented the HG-induced changes in mitochondrial morphology (Fig. 4C, D). Using DCFH-DA assay, we demonstrated that the ROS level increased after HG treatment. Simultaneously, SOD2, an ROS scavenger, was decreased (Fig. 4A, B, E–H). Mitochondrial transmembrane potential ($\Delta\Psi$) analysis confirmed that HG caused accumulation of JC-1 monomers, indicating the occurrence of decreased $\Delta\Psi$ (Fig. 4I–L). Adenosine triphosphate (ATP) concentrations were also decreased after HG exposure (Fig. 4O). In addition, HG increased HPC senescence (Fig. 4M, N). Treatment with roscovitine or knockdown Cdk5 expression reversed all these changes in mitochondria caused by HG. Taken together, these results provide evidence that the over-

activation of Cdk5 induced by hyperglycemia is an important reason for podocyte mitochondrial dysfunction and injury in diabetes.

Phospho-Sirt1 is upregulated in the glomeruli of patients with DN and human podocytes

Sirt1 is an NAD-dependent deacetylase that has a protective effect in the maintenance of mitochondrial function (3, 46). Phosphorylation of Sirt1 has negative effects on cellular functions (11), so we assessed levels of phospho-Sirt1 in diabetic glomeruli. Positive staining of p-Sirt1 was located in the nucleus of DN glomerular cells (Fig. 5A). The expression level of p-Sirt1 in DN glomeruli was significantly higher than that in control glomerulus (Fig. 5B). In contrast to p-Sirt1, the expression level of Sirt1 in DN glomeruli was significantly lower than that in control glomerulus (Fig. 5A, C). Western blotting results indicated that Sirt1 expression was significantly downregulated after HG treatment (Fig. 5D, F, Supplementary Figure S3), where p-Sirt1 level was increased 12 h after HG treatment and reached its highest levels at 24 and 48 h (Fig. 5D, E, Supplementary Figure S3).

FIG. 5. HG upregulated phosphor-Sirt1 in the glomeruli of DN patients and human podocytes. (A) Representative immunohistochemical staining for p-Sirt1 and Sirt1 in the glomerulus. Scale bars: 50 μ m. Magnification: 400 $\times$. (B, C) Quantification of p-Sirt1 and Sirt1 positive rate in the glomerulus. ($p < 0.05$, $n = 19$) (D) Protein representative Sirt1 and p-Sirt1 in HPCs induced by 30 mM glucose for different time points. (E, F) Protein levels of p-Sirt1 and Sirt1. (G, H) The expression of p-Sirt1 and Sirt1 in HG-induced HPCs by immunofluorescence at 24 h. DAPI is a nucleus dye. Scale bar: 25 μ m. The data represent the mean $\pm$ SD ($n = 3$). p-, phospho-. * $p < 0.05$ compared with the 0 h group. Please refer to Supplementary Figure S3 for raw Western blot records shown in the figure. Color images are available online.

Immunofluorescence analysis indicated that Sirt1 and p-Sirt1 were all expressed in the nuclei in podocytes. Following HG treatment, p-Sirt1 markedly expressed HG cells than in control cells, but Sirt1 expression decreased (Fig. 5G, H).

We used HG to stimulate HPCs at 0, 1, 3, 6, 12, 24, 48, and 72 h and found that although Mcl-1 expression decreased at 1, 3, and 6 h, it remained unchanged at 24 h as it was at 0 h. At the same time, we treated HPCs with resveratrol (RSV), a Sirt1 activator, or EX527, a Sirt1 inhibitor, and found that Mcl-1 expression remained the same at 24 h. In addition, we treated HPCs with Marinopyrrole A, a Mcl-1 inhibitor, and found that the expression of Sirt1 and p-Sirt1 remained unchanged. We speculate that HG promotes mitochondrial dysfunction through Cdk5-Sirt1 at 24 h, which is not associated with Mcl-1 (Supplementary Fig. S4A–J).

Downregulated phospho-Sirt1 alleviates the mitochondrial dysfunction and podocyte injury induced by HG

Studies have shown that Cdk5 regulates the phosphorylation of Sirt1 at S47 site (4). Thus, we investigated whether Cdk5 mediated the mitochondrial dysfunction of podocyte by phosphorylating Sirt1 at the same site. To further investigate the role of Sirt1 phosphorylation on podocyte mitochondrial dysfunction, we constructed a mutant plasmid of Sirt1 at S47, in which the serine was replaced with an alanine (Flag-Sirt1-S47A). The mutant Sirt1-S47A mimicked the nonphosphorylated form (Fig. 6A, Supplementary Fig. S5).

As shown in Figure 6 and Supplementary Figure S5, compared with the group transfected with pcDNA3.0, the downregulation of synaptopodin and nephrin induced by 24 h HG was significantly reversed by transfected mutant Sirt1 (S47A) (Fig. 6B–D). Drp1 expression was markedly increased with HG treatment, accompanied with p-Drp1 upregulation in transfected control cells (Fig. 6B, E, F); CytoC levels were also increased in these cells (Fig. 6B, I). In contrast, Mfn1 and SOD2 levels were significantly decreased (Fig. 6B, G, H). The mutation of Sirt1 with mutant S47A caused opposite effects on the expression of these proteins (Fig. 6B–I). MitoTracker Red staining showed that HG-induced mitochondrial swelling was alleviated in the presence of mutant Sirt1 (S47A) (Fig. 6J and Supplementary Fig. S6A). Furthermore, mutating Sirt1 (S47A) to prevent its phosphorylation also reduced ROS generation (Fig. 6K–M and Supplementary Fig. S6B) and increased $\Delta\Psi$ M (Fig. 6N, O and Supplementary Fig. S6C, D), ATP levels (Fig. 6Q), and senescence levels (Fig. 6P and Supplementary Fig. S6E).

S47 phosphorylation of Sirt1 is mediated by Cdk5 in podocytes after HG treatment

We used immunofluorescence to investigate the interaction between Cdk5 and Sirt1 in human tissues. In Figure 7A, compared with control cells, Cdk5 expression was increased in the glomeruli of DN, whereas Sirt1 was less expressed. As shown in Figure 7B, p-Sirt1 expression significantly increased, accompanied with the upregulation of Cdk5. The results also demonstrated that p-Sirt1 colocalized with Cdk5 in glomerular cells. Following HG treatment in HPCs, p-Sirt1 increased and colocalized with Cdk5 in podocyte nucleus. Contrast to p-Sirt1, the Sirt1 downregulated and colocalized with Cdk5 in podocyte nucleus (Fig. 7C, D). We performed co-immunoprecipitation

(Co-IP) experiments to verify the interaction of Cdk5 and Sirt1 in HPCs. As shown in Figure 7E and Supplementary Figure S7 and F, Cdk5 was increased by 24 h HG in Sirt1 IP, whereas Sirt1 and p-Sirt1 were also increased in Cdk5 IP.

To explore the effect of Cdk5 on Sirt1 phosphorylation, we first transfected HPCs with WT-Cdk5, the wild-type plasmid of Cdk5. The results showed that WT-Cdk5 plasmid increased the expression and kinase activity of Cdk5 (Fig. 8A, D, E) and upregulated the expression level of p-Sirt1 in HPCs (Fig. 8H, I). To further detect the kinase activity of Cdk5 on p-Sirt1 expression, p25 plasmid, which could overactivate Cdk5, was transfected into HPCs (Fig. 8B, C). Aberrant activation of Cdk5 *via* overexpressed p25 also increased the phosphorylation level of Sirt1 in HPCs (Fig. 8F, G). To determine the Cdk5 phosphorylation site on Sirt1, the mutant Sirt1 plasmid (Sirt1-S47A) was cotransfected with WT-Cdk5 or p25 plasmid. We demonstrated that Sirt1-S47A decreased the Cdk5-induced phosphorylation of Sirt1 in HPCs (Fig. 8F–I). Furthermore, the kinase activity and mRNA expression of Cdk5 were inhibited by roscovitine and DN-Cdk5, respectively. The results showed that inhibition of Cdk5, whether its kinase activity or mRNA expression, significantly reduced p-Sirt1 expression induced by HG (Fig. 8J, K).

In normal podocytes transfected with WT-Cdk5, we observed a marked decrease in nephrin and synaptopodin expression, which was reversed by cotransfection with Sirt1-S47A. Immunoblot analyses revealed that the upregulation of Drp1, p-Drp1, and Cyto C, and the downregulation of Mfn1 and SOD2 induced by WT-Cdk5 were also reversed by Sirt1-S47A (Fig. 8L, M, Supplementary Fig. S8). In addition, mitochondrial swelling, increased ROS, increased senescence, decreased ATP, and the loss of mitochondrial voltage potential induced by Cdk5 overexpression were also attenuated by Sirt1-S47A (Fig. 8N–U and Supplementary Fig. S9). As shown in Figure 8V and W, we verified that ROS was involved in Cdk5-mediated podocyte injury. Thus, these results suggest that Cdk5 promotes Sirt1 phosphorylation at S47, leading to mitochondrial dysfunction and podocyte damage.

Cdk5 downregulation by shRNA lentivirus attenuates podocyte injury in diabetic mice

Our finding demonstrated that Cdk5 inhibition might be a valuable therapeutic approach for DN. We used lentivirus vectors to deliver Cdk5 shRNA to STZ-induced type 1 diabetic mice or type 2 db/db mice. A significant reduction in the glomerular expression of Cdk5 was detected in the mice treated with lentivirus-Cdk5 shRNA, with subsequent significant recovery of synaptopodin and nephrin expression (Fig. 9E G and Supplementary Figs. S10E, F and S11). Increased expression of Sirt1 and decreased p-Sirt1 were also observed in lentivirus-Cdk5 shRNA-infected mice (Fig. 9F, G and Supplementary Fig. S10E, G, H). To evaluate the effects of Cdk5 shRNA on mitochondrial dynamics, the mitochondrial fission and fusion proteins were also assessed. Drp1 and p-Drp1 levels were downregulated and Mfn1 was upregulated (Supplementary Fig. S11); and the abnormal increase in CytoC expression was restored by lentivirus-Cdk5 shRNA treatment in diabetic mice (Supplementary Fig. S11).

Furthermore, transmission electron microscopy (TEM) results confirmed the alleviation of podocyte FPs effacement, glomerular basement membrane thickening, and mitochondrial swelling in lentivirus-Cdk5 shRNA-treated mice compared with diabetic mice (Fig. 9B–D and Supplementary

FIG. 6. Downregulated the phospho-Sirt1 alleviated the HG-induced mitochondrial dysfunction and podocyte injury at 24h. (A) Transfection efficiency of Flag-Sirt1-S47A. (B) Protein representative synaptopodin, nephrin, Drp1, p-Drp1, Mfn1, SOD2, and CytoC in HPCs. (C–I) Protein levels of synaptopodin, nephrin, Drp1, p-Drp1, Mfn1, SOD2, and CytoC. (J) The MitoTracker Deep Red FM (50 nM) was visualized under confocal microscopy and it was quantified. (K) The MitoSOX Red was visualized under confocal microscopy and it was quantified. (L, M) ROS generation was measured by flow cytometry and was quantified following DCFH-DA (10 μ M) staining. (N) The mitochondrial membrane potential in HPCs was measured by flow cytometry and was quantified following JC-1 (100 μ M) staining. (O) The quantitative analysis of SA- β -gal staining in HPCs. (P) ATP concentrations were determined using an ATP determination kit. The data represent the mean $\pm$ SD ($n=3$). C, control; p-, phospho-. * $p < 0.05$ compared with the control group, # $p < 0.05$ compared with the HG+pcDNA3.0 group. Scale bar: 10 μ m. Please refer to Supplementary Figure S5 for raw Western blot records shown in the figure. Color images are available online.

FIG. 6. (Continued).

FIG. 7. The interaction between Cdk5 and Sirt1. (A, B) Colocalization of Cdk5 with Sirt1 and p-Sirt1 in DN kidney tissues by immunofluorescence. DAPI is a nucleus dye. Scale bars: 50 μm. Magnification: 400×. (C, D) Colocalization of Cdk5 with Sirt1 and p-Sirt1 in the HPCs treated with HG (30 mM) by immunofluorescence at 24 h. DAPI is a nucleus dye. Scale bars: 25 μm. (E) Co-IP analysis demonstrated that Cdk5 protein in Sirt1 precipitates was enhanced by HG (30 mM) at 24 h. (F) Co-IP analysis demonstrated that Sirt1 and p-Sirt1 protein in Cdk5 precipitates were enhanced by HG (30 mM) at 24 h. C, control; p-, phospho-. Please refer to Supplementary Figure S7 for raw Western blot records shown in the figure. Color images are available online.

Fig. S10B–D). Finally, analyses of biochemical parameters showed that urinary albumin, serum creatinine, and blood urea nitrogen (BUN), which were notably increased in diabetic mice, were decreased in lentivirus-Cdk5 shRNA-treated mice (Fig. 9A and Supplementary Fig. S10A).

Discussion

Mitochondria are the primary organelles of cell energy production, and their dysfunction is an important reason for podocyte injury in DN (40, 41, 49). The mechanism of mitochondrial dysfunction in podocytes in diabetes is not fully understood. In the current study, we demonstrated that Cdk5-mediated Sirt1 phosphorylation contributes to mitochondrial dysfunction in podocytes in diabetic mice and cell models.

Cdk5 is a proline-directed serine/threonine protein kinase that is expressed in podocytes and plays an important role in the maintenance of their normal function (44). Knockdown of Cdk5, or its activator, p35, results in podocytes apoptosis (7, 30, 57). Although many studies have demonstrated that deregulated Cdk5 is implicated in neurodegenerative diseases, such as AD and PD, the role of aberrant Cdk5 in podocyte injury has not been thoroughly investigated (19, 54). In this study, we found an upregulation in the expression of Cdk5 in the podocytes of diabetic mice and patients with DN, which

negatively correlated with nephrin and synaptopodin expressions. The expression of nephrin and synaptopodin was decreased with HG treatment in podocytes and was recovered by Cdk5 inhibitor roscovitine. The result demonstrated that Cdk5 overactivated by HG is an important reason for podocyte injury in diabetes.

The kidney is one of the most energy-demanding organs and requires a lot of mitochondria to provided sufficient energy to maintain its normal function (13). Mitochondrial homeostasis imbalance and energetics damage contribute to the progression of kidney diseases, such as DN (51). Hyperglycemia is the initiating factor and causes of mitochondrial structural and functional damages in the progression of DN, especially in podocyte injury (21). The present study confirmed that HG treatment dramatically damaged the mitochondria in podocytes. We observed significant mitochondrial swelling and fragmentation following a HG treatment. Mitochondria are highly dynamic organelles and constantly undergoing fission and fusion to maintain the mitochondrial homeostasis in physiological conditions (16). In diabetic conditions, the mitochondrial fission in podocytes significantly increased (32). Cdk5 mediates mitochondrial fission in neurodegenerative diseases (18). The current study showed that inhibition of Cdk5 by roscovitine or shRNA significantly decreased the number of fragmented

FIG. 8. S47 phosphorylation of Sirt1 is mediated by Cdk5 in podocytes after HG treatment. (A) Transfection efficiency of WT-Cdk5. (B–E) Western blot assay showed the Histone H1 and phospho-Histone H1 expression in HPCs after transfected with the WT-Cdk5 and P25 plasmid. * $p < 0.05$ compared with the pcDNA3.0 group. (F, G) Western blot assay showed the Sirt1 and p-Sirt1 expression in HPCs after transfected with the P25 and Sirt1-S47A plasmid. * $p < 0.05$ compared with the pcDNA3.0 group. # $p < 0.05$ compared with the P25 group. (H, I) Western blot assay showed the Sirt1 and p-Sirt1 expression in HPCs after transfected with the WT-Cdk5 and Sirt1-S47A plasmid. * $p < 0.05$ compared with the pcDNA3.0 group. # $p < 0.05$ compared with the WT-Cdk5 group. (J, K) Protein representative Sirt1 and p-Sirt1 in HPCs, and quantitative analyses of the results from Western blot. * $p < 0.05$ compared with the WT-Cdk5 group. # $p < 0.05$ compared with the HG group. (L, M) Protein representative synaptopodin, nephrin, Drp1, p-Drp1, Mfn1, SOD2, and CytoC in HPCs, and quantitative analyses of the results from Western blot. (N) The MitoTracker Deep Red FM (50 nM) was visualized under confocal microscopy and it was quantified. (O, P) The mitochondrial membrane potential was quantified following JC-1 (100 μ M) staining. (Q) The ROS generation was measured by flow cytometry and was quantified following JC-1 (100 μ M) staining. (R) The mitochondrial membrane potential was measured by JC-1 (100 μ M) staining. (T) ATP concentrations were determined using an ATP determination kit. (U) The quantitative analysis of SA- β -gal staining in HPCs. (V, W) Western blot assay showed the effects of NAC, an antioxidant, on expression of synaptopodin and nephrin in HPCs transfected with WT-Cdk5 plasmid. The data represent the mean $\pm$ SD ($n = 3$). C, control; p-, phospho-. figure. NAC, N-acetyl-L-cysteine. Color images are available online.

FIG. 8. (Continued).

FIG. 9. Lentivirus-Cdk5 shRNA attenuated podocyte injury in diabetic mice. (A) Urinary albumin (mg/L), serum creatinine (μ M), and blood urea nitrogen (mM) of db/db mice. (B–D) Transmission electron microscopy assay showed the ultrastructure of the podocytes in renal cortex of db/db mice. Red lines referred to the thickness of the GBM, and red arrows referred to mitochondria of podocytes. Scale bar: 500 nm. Magnification: 20,000 $\times$. The data represent the mean $\pm$ SD ($n=5$). * $p<0.05$ (E–G) Immunohistochemical staining of Cdk5, Sirt1, and p-Sirt1 in the glomerulus from db/db mice. Scale bars: 50 μ m. Magnification: 400 $\times$. The data represent the mean $\pm$ SD ($n=5$). GBM, glomerular basement membrane; p-, phospho-. Color images are available online.

mitochondria and elongated it. Drp1 and Mfn1 are the major proteins that control mitochondrial fission and fusion. Wang *et al.* showed that ROCK1 promotes mitochondrial fission by recruiting Drp1 to the mitochondria in diabetic mice (47). Phosphorylation of Drp1 also promotes mitochondrial fission and DN progression (17, 50). In our study, the expression of Drp1 and phosphorylated Drp1 was significantly increased and that of Mfn1 decreased following a HG treatment, whereas these changes were reversed by Cdk5 suppression. Hyperglycemia promotes ROS generation, which caused the decrease in ATP production, the release of Cyto C, and the dysfunction of mitochondria (22, 35). Inhibition of Cdk5 also effectively reduced ROS production and CytoC release in HG-treated podocyte and rescued ATP generation and $\Delta\Psi$ M.

Sirt1 is an NAD⁺-dependent deacetylase that belongs to the sirtuin family (34). Sirt1 participates in mitochondrial energy metabolism through deacetylation of PGC-1 α (9). There is a growing literature describing the activation or upregulation of Sirt1 protecting DN from inflammation, apoptosis, and fibrosis (14, 43, 48). It has been shown that Sirt1 protects podocyte against the damage of hyperglycemia in diabetes mellitus. Podocyte-specific Sirt1 deletion leads to increased urinary protein level and the aggravation of renal injury (20, 29). Our study also showed the decreased expression of Sirt1 in podocytes following a HG treatment.

Sirt1 activators, such as RSV and other synthetic drugs, have not been shown definitively to have beneficial effects on DN in clinical trials. There are differences in the effects of Sirt1 activators between clinical and preclinical studies. Post-translational modification is an important method to regulate the activity of Sirt1. Phosphorylation regulation can significantly reduce Sirt1 activities (12). We surmise that intervention from the perspective of post-translational modification may enhance Sirt1 activity and its therapeutic effects on DN. During the progression of podocyte injury *in vivo* and *in vitro*, the phosphorylation of Sirt1 was significantly upregulated at S47, leading to mitochondrial dysfunction. Inhibition of Sirt1 at S47 by mutating the single amino acid ameliorates the podocyte injury, mitochondrial dynamic and mitochondrial dysfunction in HG-treated podocyte. This demonstrated that increased phosphorylation of Sirt1 caused mitochondrial dysfunction and leads to podocyte damage.

Overactivated Cdk5 leading to more phosphorylation of its target substrates is an important underlying factor for several diseases, such as AD and PD (36, 40, 56). Also, Cdk5 was reported to phosphorylate Sirt1 and inhibit its antisenescent and anti-inflammatory activities (4). The present study confirmed that overactivated Cdk5 upregulated the level of phosphorylated Sirt1 at S47. Suppression of Cdk5 down-regulated the Sirt1 phosphorylation level, and the nonphosphorylation mutation form S47A abolished the mitochondrial dysfunction and podocyte injury induced by WT-Cdk5 plasmid. These results indicate that Cdk5 overactivation promotes Sirt1 phosphorylation is an important reason for mitochondrial dysfunction and podocyte injury in DN.

In *in vitro* study, knockdown of Cdk5 expression by lentivirus-Cdk5 shRNA significantly attenuated podocyte injury. From our points of view, Cdk5 interference may enhance the therapeutic effect of RSV and other Sirt1 activators by increasing Sirt1 activity through post-translational modifications. Thus, Cdk5-Sirt1 signaling pathway may be a valuable therapeutic target for DN.

FIG. 10. Mechanistic depiction of the role of Cdk5 in podocyte mitochondrial dysfunction. Our study reveals foremost that Cdk5 as critical kinase by promoting Sirt1 phosphorylation at S47 participates in mitochondrial dysfunction and podocyte injury, which leads to the progression of diabetic nephropathy. Color images are available online.

The current study firmly established that Cdk5, as a critical kinase that promotes Sirt1 phosphorylation at S47, participates in mitochondrial dysfunction and podocyte injury, which leads to the progression of DN. Inhibition of Cdk5 activities may be a potential target to reverse podocyte injury and reduce the progression of DN (Fig. 10).

Materials and Methods

Reagents

McCoy's 5A (Modified) Medium and fetal calf serum were purchased from Gibco (Carlsbad, CA). D-glucose was obtained from Solarbio (Beijing, China). FuGENE HD Transfection Reagent was purchased from Promega (Madison, WI). Antibodies against Cdk5 (ab40473), Drp1 (ab184247), nephrin (ab58596), and Sirt1 (ab110304) were purchased from Abcam (Cambridge, United Kingdom). Antibodies for β -actin (20536-1-AP), Mfn1 (13798-1-AP), and synaptopodin (21064-1-AP) were obtained from Proteintech Group (Wuhan, Hubei, China). Antibodies for p-Sirt1 (2314), p35/25 (2680), and p-Drp1 (3455) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against SOD2 (EM1717-32) and Cdk5 (SR39-01) were purchased from HuaBio Technology (Hangzhou, Zhejiang, China). WT-Cdk5, DN-Cdk5, and Sirt1 plasmids were obtained from Addgene (Watertown, MA). MitoTracker™ Deep Red FM and MitoSOX™ Red were purchased from Thermo Fisher (Waltham, MA). DCFH-DA was purchased from Sigma (St. Louis, MO). JC-1 was purchased from Cayman Chemical (Ann Arbor, MI). Roscovitine and STZ were obtained from Sigma (Shanghai, China). Enhanced ATP Assay Kit was obtained from Beyotime (Shanghai, China). Biochemical detection kits for 24h urinary protein, BUN, and serum

creatinine (Scr) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Mut Express II Fast Mutagenesis Kit V2 was obtained from Vazyme (Nanjing, Jiangsu, China). Senescence β -Galactosidase Staining Kit (C0602) was purchased from Beyotime (Shanghai, China). Takara (Shiga, Japan) was the source of SYBR Premix Ex Taq II.

Human renal specimens

Nineteen patients aged 30–55 years diagnosed with DN were recruited from the Inpatient Department of Nephrology at the Second Hospital of Hebei Medical University from 2017 to 2018. The study protocols concerning human tissues are consistent with the principles of the Declaration of Helsinki and were approved by the Clinical Research Ethics Committee of Hebei Medical University (IRB number: 20190040). Nineteen samples of control kidney tissues were acquired from nephrectomies performed for the treatment of renal lipomyoma and pathologically confirmed normal kidney tissues without history of other autoimmune diseases, DN, hypertension nephropathy, or severe infections. Informed consent was obtained from each patient, and patients' clinical biochemical data were extracted from medical records. Assessment of the histopathology was evaluated in a blinded manner by two experienced pathologists and classified according to the Renal Pathology Society's Pathologic Classification of DN based on glomerular pathology. Kidney tissues were fixed with 4% formaldehyde for hematoxylin and eosin, periodic acid–Schiff, and immunohistochemical staining.

Animal models

All animal studies were in accordance with the guidelines from the Care and Use of Laboratory Animals and were approved by the Animal Care and Ethics Committee of Hebei Medical University (approval ID: HebMU 20080026). The mice were housed in an air-conditioned room at 24°C with alternating 12 h cycles of light and dark. All mice were maintained on a standard diet (SPF mice maintain feed; Beijing Keao Xieli Feed Co., Ltd.) and given tap water *ad libitum*.

STZ mice

Eight-week-old male CD1 mice (30–40 g) were obtained from Beijing Charles River Laboratory Animal Technology Co. Ltd (Beijing China). After 7 days of adaptation, 24 CD1 mice were randomly divided into 3 groups: control group (8 mice), STZ group (8 mice), and STZ+Lv-Cdk5shRNA group (8 mice). Mice in the STZ group and the STZ+Lv-Cdk5shRNA group were induced by a single intraperitoneal injection of STZ at 150 mg/kg. Control mice received an equal volume of vehicle (0.1 M citrate buffer, pH 4.5). Individual animals with blood glucose concentrations of 16.7 mM for three consecutive days were confirmed as diabetic mice (31).

C57BLKS/J db/db mice

Ten 8-week-old male C57BLKS/J db/db mice (32–36 g) with genetic type 2 diabetes and 5 healthy m/m male mice (26–28 g) were purchased from GemPharmatech Co., Ltd. (Nanj-

ing, China). The 10 db/db mice were randomly divided into 2 groups: db/db group ($n = 5$) and db/db+Lv-Cdk5shRNA group ($n = 5$). Five control mice served as the m/m group.

To verify the protective effect of Cdk5 inhibition on kidney *in vivo*, eight STZ+Lv-Cdk5shRNA mice and five db/db+Lv-Cdk5shRNA mice were renally injected with $50 \mu\text{L}$ 1×10^6 infective units of lentivirus-Cdk5 shRNA in both kidneys at 24 weeks. Mice in the control group and the m/m group were injected with isometric saline. At the end of 4 weeks after treatment, mice were kept in individual metabolic cages for 24 h urine collection to measure urine albumin. The mice were anesthetized with 5% chloral hydrate, and the blood samples were collected for blood glucose, BUN, and serum creatinine (Scr). Then, the mice were sacrificed, and the kidneys were harvested immediately for relevant investigations.

Plasmid and lentivirus construction

Sirt1 site-directed mutagenesis was performed to generate a construct in which S47 was replaced with alanine (Flag-Sirt1-S47A) (Mut Express II; Vazyme). All steps are according to the manufacturer's instructions. Transient transfection was performed in human podocytes with FuGENE HD transfection reagent, and overexpression was confirmed by Western blotting using the anti-Flag antibody.

For *in vivo* inhibition, the lentivirus suppressing Cdk5 expression was produced with the short hairpin (sh) RNA sequence 5'-CGGGAGATCTGTCTACTCAAACCTCGA GTTTGAG TAGACAGATCTCCCG-3' by Cyagen (Santa Clara, CA, USA). A scrambled shRNA was used as the negative control.

Cell culture and transfection

The primary HPCs purchased from BeNa Culture Collection (BNCC, Beijing, China) were cultured in McCoy's 5A Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin at 37°C in a 5% CO₂ atmosphere. HPCs were transiently transfected at 10^6 cells per well using FuGENE HD Transfection Reagent (Promega) according to the manufacturer's instructions. Briefly, the reagent was premixed with the various plasmids at a ratio of 1.0 μg DNA to 3 μL transfection reagents in serum-free DMEM and incubated for 15 min at room temperature, and then, the mixture was added to the cells cultured in six-well plates. After 6 h, the medium was replaced with normal culture medium and incubated for another 24 h. Western blotting was used to detect the transfection efficiency.

Western blotting and Co-IP

Proteins were extracted from HPCs using RIPA buffer (Solarbio, Beijing, China), separated by 10% SDS-PAGE, and transferred to a PVDF membrane (Millipore, Burlington, MA). The membrane was blocked with 5% nonfat milk and incubated with the primary antibodies overnight at 4°C. After washing with TBS-T buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 0.5% Tween 20), the membranes were incubated with goat anti-rabbit or mouse secondary antibody conjugated to horseradish peroxidase. After washing, bands

were detected by ECL reagent and scanned using the Odyssey Fc System (LI-COR, Lincoln, NE).

For Co-IP, cells were lysed with lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% NP-40, 1 mM PMSF, 2 mg/mL aprotinin, pH 7.4) on ice for 30 min and centrifuged at 12,000 rpm for 20 min. The supernatant was incubated with the appropriate primary antibodies overnight and followed by the addition of protein A/G-Sepharose (Santa Cruz, Dallas, TX) at 4°C for 3 h. After three washes with homogenization buffer, the beads were boiled with a 6×buffer for 5 min followed by SDS-PAGE and Western blotting using the appropriate antibodies. The protein bands were detected by ECL detection system.

RT-qPCR analysis

We extracted total RNA from HPCs by using TRIzol reagent and RT-qPCR kits according to the manufacturer's guidelines. Complementary DNA was amplified with specific primers by means of PCR for nephrin, synaptopodin, and 18s rRNA. SYBR Premix Ex Taq II was used to perform real-time PCR, and real-time PCRs were processed on an Agilent Mx3000P qPCR Systems (Agilent, CA). Primers were as follows: human synaptopodin (forward 5'-CTTACGGC GGTGACATCTC-3', reverse 5'-GGTCCTGAGCCTCGA TCC-3') and human nephrin (forward 5'-AGCTCGTG TCTCCCAGAGT-3', reverse 5'-CGTTCACGTTTGCAG AGATGT-3').

In vitro Cdk5 kinase activity assay

The protein G Plus/protein A-agarose beads with Cdk5 protein was incubated with 6 μL of assay dilution buffer containing 10 μg of histone H1 (14–155; Millipore Sigma, Burlington, MA) and 12 μL of magnesium/ATP mixture (20–113; Millipore Sigma) in a final volume of 30 μL at 30°C for 30 min. The binding strength of phospho-histone H1 was assessed by immunoblotting with specific anti-phospho-histone H1 antibody (06–597; Millipore Sigma) (31).

Mitochondrial morphology

The mitochondrial morphology was observed by MitoTracker Deep Red FM staining. After treatment, cells were incubated with prewarmed (37°C) staining solution (50 nM) for 30 min at 37°C in the dark. The stained cells washed with prewarmed phosphate-buffered saline (PBS) buffer and observed with a laser confocal microscope (Leica, Germany).

Mitochondrial ROS detection and flow cytometry

The mitochondrial formation of ROS was detected using MitoSOX Red. After culture in six-well plates under the different experimental conditions for 48 h, cells were incubated with MitoSOX Red at a final concentration of 5 μM for 30 min at 37°C. Cells were washed twice with PBS, and images were acquired using a laser confocal microscope (Leica). The mitochondrial ROS intensity was quantified using the Image-Pro Plus 6.0 software.

For flow cytometry, cells were incubated with DCFH-DA probe (10 μM) for 30 min at 37°C in the dark incubator, analyzed with an FACS flow cytometer (FACS Aria, BD Biosciences, CA) at specific excitation and emission wave-

lengths (488 and 525 nm, respectively). The mean fluorescence intensity (MFI) of $\sim 1 \times 10^4$ cells was analyzed using the FlowJo software (27).

$\Delta\Psi_m$ assay

Cells were seeded on a six-well plate, treated for indicated time, and then washed twice with PBS before incubation with 5 μM JC-1 probe at 37°C in the dark for 30 min. After thorough washing, the cells were subjected to FACS flow cytometer or detected by a fluorospectrophotometer. Results of flow cytometry were analyzed using the FlowJo software (53). Images were acquired using a laser confocal microscope (Leica, Germany). The $\Delta\Psi_m$ intensity was quantified using the Image-Pro Plus 6.0 software.

Intracellular ATP content determination

ATP levels were assessed using an ATP Determination Kit (Beyotime Company, China). For this, cells were harvested and lysed with lysis reagent. After centrifugation at 12,000 g for 20 min at 4°C, the supernatant was collected, and the ATP levels were detected according to the manufacturer's instructions. Cellular ATP levels were expressed as a percentage of the levels observed in control cells.

Senescence β -galactosidase staining

Detection of SA- β -Gal-positive cells was conducted using a kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, HPCs were seeded in six-well plates at a cell density of 5.0×10^4 cells per well containing complete medium and incubated at 37°C for 24 h. Cells were washed in PBS and fixed with 2% formaldehyde and then incubated with SA- β -gal staining solution at 37°C. After 24 h incubation, the “blue” cells expressing SA- β -Gal were counted under the microscope.

Immunohistochemistry

Immunohistochemistry for antibodies on renal sections was performed with SP kit according to the instructions of the manufacturer. Paraffin-embedded tissue sections (4 μm) were deparaffinized in xylene and rehydrated with graded ethanol. Internal peroxidase was inactivated with 3% hydrogen peroxide in 100% methanol for 30 min. Antigen retrieval was subsequently performed by autoclaving for 5 min at 121°C in a sodium citrate buffer (pH 6.0). After blocking with 10% normal goat serum for 30 min at 37°C, the sections were incubated with primary antibodies for Cdk5, nephrin, synaptopodin, Sirt1, p-Sirt1, Drp1, p-Drp1, Mfn1, or CytoC overnight at 4°C. The next day, sections were washed with PBS and incubated with secondary antibody for 30 min at 37°C. Labeling was visualized with 3,3-diaminobenzidine to produce a brown color, and sections were counterstained with hematoxylin.

Immunofluorescence

Human podocytes were grown and stimulated in six-well chamber slides and fixed with frozen acetone for 10 min at 4°C. The cell membranes were punched with Triton X-100 before incubation with anti-Cdk5, anti-nephrin, anti-synaptopodin, anti-Sirt1, or anti-p-Sirt1 antibody at 4°C

overnight. The cells were then exposed to goat anti-rabbit Alexa Fluor 552-conjugated secondary antibody for 1 h at 37°C. Immunofluorescence of cells was then examined by laser confocal microscopy.

Transmission electron microscopy

Renal tissues of diabetic mice were perfused with PBS and cut into 1 mm³ blocks, which were immediately fixed with 4% glutaraldehyde. After embedding in epoxy resin, the ultrathin tissue sections were stained with uranyl acetate and lead citrate and then examined with Hitachi H7650 transmission electron microscope and photographed. Images were analyzed with the ImageJ software. Quantification of mitochondria was performed as described previously. The morphology of a total 100 mitochondria was examined for all the groups.

Statistical analysis

All experiments were performed at least three separate times, with data obtained from triplicate wells in each experiment. Data are expressed as means ± SD. The statistical difference between the two groups was analyzed by an unpaired Student's *t*-test (two-tailed); multiple groups were compared using one-way analysis of variance and correlated by the Spearman correlation analysis (GraphPad Prism 5.0, Bethesda, MD). A value of *p* < 0.05 was considered significant.

Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5
Supplementary Figure S6
Supplementary Figure S7
Supplementary Figure S8
Supplementary Figure S9
Supplementary Figure S10
Supplementary Figure S11

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Abbreviations Used

$\Delta\Psi$	= mitochondrial transmembrane potential
AD	= Alzheimer's disease
ATP	= adenosine triphosphate
BUN	= blood urea nitrogen
Cdk5	= cyclin-dependent kinase 5
Co-IP	= co-immunoprecipitation
DN	= diabetic nephropathy
FPs	= foot processes
GBM	= glomerular basement membrane
HG	= high glucose
HPC	= human renal podocyte
NAC	= N-acetyl-L-cysteine
PBS	= phosphate-buffered saline
PD	= Parkinson's disease
ROS	= reactive oxygen species
RSV	= resveratrol
RT-qPCR	= quantitative real-time polymerase chain reaction
Sirt1	= Sirtuin 1
STZ	= streptozotocin
TGF- β 1	= transforming growth factor- β 1