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Original Paper

Hyperglycaemia Stress-Induced Renal Injury is Caused by Extensive Mitochondrial Fragmentation, Attenuated MKP1 Signalling, and Activated JNK-CaMKII-Fis1 Biological Axis

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Key Words
Diabetic nephropathy • Mitochondrial fragmentation • MKP1 • JNK-CaMKII-Fis1 pathway

Abstract
Background/Aims: Hyperglycaemia stress-induced renal injury is closely associated with mitochondrial dysfunction through poorly understood mechanisms. The aim of our study is to explore the upstream trigger and the downstream effector driving diabetic nephropathy via modulating mitochondrial homeostasis. ***Methods:*** A diabetic nephropathy model was generated in wild-type (WT) mice and MAP Kinase phosphatase 1 transgenic (MKP1-TG) mice using STZ injection. Cell experiments were conducted via high-glucose treatment in the human renal mesangial cell line (HRMC). MKP1 overexpression assay was carried out via adenovirus transfection. Renal function was evaluated via ELISA, western blotting, histopathological staining, and immunofluorescence. Mitochondrial function was determined via mitochondrial potential analysis, ROS detection, ATP measurement, mitochondrial permeability transition pore (mPTP) opening evaluation, and immunofluorescence for mitochondrial pro-apoptotic factors. Loss- and gain-of-function assays for mitochondrial fragmentation were performed using a pharmacological agonist and blocker. Western blotting and the pathway blocker were used to establish the signalling pathway in response to MKP1 overexpression in the presence of hyperglycaemia stress. ***Results:*** MKP1 was downregulated in the presence of chronic high-glucose stress *in vivo* and *in vitro*. However, MKP1 overexpression improved the metabolic parameters, enhanced glucose control, sustained renal function, attenuated kidney oxidative stress, inhibited the renal inflammation response, alleviated HRMC apoptosis, and repressed tubulointerstitial fibrosis. Molecular investigation found that MKP1 overexpression enhanced the resistance of HRMC to the hyperglycaemic injury by abolishing mitochondrial fragmentation. Hyperglycaemia-triggered mitochondrial fragmentation promoted mitochondrial dysfunction,

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as evidenced by decreased mitochondrial potential, elevated mitochondrial ROS production, increased pro-apoptotic factor leakage, augmented mPTP opening and activated caspase-9 apoptotic pathway. Interestingly, MKP1 overexpression strongly abrogated mitochondrial fragmentation and sustained mitochondrial homeostasis via inhibiting the JNK-CaMKII-Fis1 pathway. After re-activation of the JNK-CaMKII-Fis1 pathway, the beneficial effects of MKP1 overexpression on mitochondrial protection disappeared. ***Conclusion:*** Taken together, our data identified the protective role played by MKP1 in regulating diabetic renal injury via repressing mitochondrial fragmentation and inactivating the JNK-CaMKII-Fis1 pathway, which may pave the road to new therapeutic modalities for the treatment of diabetic nephropathy.

Introduction

Diabetes mellitus is the most common cause of chronic kidney dysfunction, which is defined as diabetic nephropathy in the clinic. Diabetic nephropathy, also called nodular diabetic glomerulosclerosis, is primarily diagnosed by heavy proteinuria, irreversible glomerular filtration rate reduction, and renal fibrosis [1]. According to previous studies, approximately 25% to 45% of patients with diabetes develop nephropathy after 20 years of the disease [2]. Despite currently established therapy, diabetic nephropathy remains a leading cause of hospitalization and mortality worldwide [3]. More importantly, follow-up clinical studies show that intensive glucose control at the time of diabetes diagnosis could not protect against nephropathy complications in early and post-trial stages due to the existence of hypertension, hyperlipidaemia, microvascular damage and other residual metabolism disorders. Accordingly, in-depth knowledge about the molecular features underpinning the hyperglycaemia stress-mediated renal damage is urgent to design effective approaches to slow the progression of diabetic nephropathy.

Disrupted mitochondrial quality control has been noted in the pathogenic process of hyperglycaemia stress-mediated renal damage [4, 5]. For example, mitochondrial respiration is repressed in hyperglycaemia-cultured renal mesangial and tubular cells [6]. Damaged mitochondria release the mitochondrial DNA into blood, which has been acknowledged as a potential noninvasive biomarker of the severity of diabetic nephropathy [7]. Dysregulated mitochondrial bioenergetics also participates into the development of hyperglycaemia-associated renal damage [8]. Excessive accumulation of damaged mitochondria due to mitophagy arrest also promotes the renal dysfunction [9]. Moreover, mitochondrial oxidative stress, mitochondrial calcium imbalance, mitochondrial antioxidant system malfunction, and oxidative phosphorylation cessation [10, 11] are the inter-related risk factors for mitochondrial aetiology in diabetic renal disorders. Recently, mitochondrial fragmentation, which primarily results from increased mitochondrial mitotic fission and/or decreased mitochondrial fusion, is found to be correlated with a fall in myocardial function in the setting of hyperglycaemia stress [12]. At the molecular level, mitochondrial fragmentation is the defensive reaction of mitochondria upon hyperglycaemia exposure. Moderate mitochondrial division helps mitochondria to remove the damaged parts and ensure the homeostasis of the mitochondrial network [13]. However, uncontrolled mitochondrial division, as evidenced by extensively fragmented mitochondria, represses the ability of mitochondria to produce sufficient ATP and finally contribute to the opening of the mitochondrial apoptotic pathway [14, 15]. Although mitochondrial fragmentation is observed in hyperglycaemia-challenged cardiomyocytes [12], the role of mitochondrial fragmentation is not defined in diabetic renal damage.

The factors leading to the activation of MAPKs have been acknowledged as the pathogenetic signalling for the hyperglycaemia-attacked kidney. MAPK-JNK activation augments podocyte apoptosis in diabetic nephropathy mice [16]. In contrast, inhibition of JNK phosphorylation improves renal function in diabetic rats [17]. Notably, a subgroup of enzymes within the PTP superfamily, termed dual specificity phosphatases, are recently identified as upstream inhibitors of the MAPK pathway, which are now named the MAPK

phosphatases (MKPs)[18]. To date, 10 typical MKPs have been characterized, and MKP1 has been documented to have the ability to promote JNK inactivation. Initial research confirms that MKP1 plays a key role in regulating nephrogenesis via modification of the JNK pathway [19]. A following study shows that MKP1 deficiency induces JNK activation and thus causes tubular cell damage in response to renal oxidative stress [20]. Another study also reports that MKP1 upregulation benefits mesangial cells and therefore represses hyperglycaemia-mediated cell apoptotic index via modification of the p38 pathway *in vitro* [21]. However, the detailed action of MKP1 in diabetic renal damage has not been explored, and whether MKP1 is involved in diabetic nephropathy development and progression via modulating mitochondrial fragmentation remains uncertain. Previous studies have suggested that mitochondrial fragmentation is primarily regulated by JNK activation and CaMKII phosphorylation [22]. The activated JNK-CaMKII pathway elevates the factors related to mitochondrial fission, such as Drp1 and Fis1, obligating mitochondria to undergo division [23]. However, it is still unknown whether MKP1 regulates hyperglycaemia-initiated mitochondrial fragmentation via JNK-CaMKII pathway. Collectively, the aim of our study is to explore the role of MKP1 in diabetic nephropathy and determine whether MKP1 overexpression protects renal function via attenuating mitochondrial fragmentations and normalizing the JNK-CaMKII pathway.

Materials and Methods

Animal treatment

Sixty male wild-type (WT) mice and MKP1 transgenic (MKP1-TG) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). These mice (six weeks old) were first housed for two weeks of acclimation in a temperature-controlled room under 12/12 h light/dark cycle and had free access to food and water. To establish the diabetic nephropathy model, freshly prepared STZ was intraperitoneally injected at a single dose of 60 mg/kg according to a previous study [24]. Four weeks later, fasting blood glucose was measured to validate the diabetes [25]. The mice with diabetes (blood glucose > 14 mM) were used in the present study (n=6 for each group)[24]. At the end of treatment, all mice were euthanized, and kidneys were collected for further experimentations. This work was performed in accordance with the approval by the Southern Medical University committee.

Measurement of metabolic parameters

Body weight and kidney weight were measured according to a previous study. Fasting glucose was evaluated via an Omron HEA-230 Glucometer (Omron Corporation, Kyoto, Japan) using the tail blood. Levels of insulin and glucagon were measured via ELISA kit obtained from Cusabio Technology (Wuhan, China). The concentration of inflammation factors such as TNFα, MCP-1 and IL-6 were determined via ELISA (Cusabio Technology, Wuhan, China). The renal function was measured via analysing the levels of creatinine and blood urea nitrogen (BUN) using a Cobas® C311 Autoanalyzer (Roche Diagnostics, Indianapolis, USA) as per the manufacturer's protocols [26].

Oxidative stress detection

Glutathione (GSH, Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalog No. T10095), glutathione peroxidase (GPX, (Beyotime Institute of Biotechnology, China; Catalog No. S0056) and SOD (Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalog No. BMS222TEN) were measured according to the manufacturer's instructions using a microplate reader (Epoch 2; BioTek Instruments, Inc.)[27].

Kidney histology

After treatment, the kidneys were isolated and then washed with PBS to remove the red blood cells. Next, the samples were fixed using 4% paraformaldehyde for 30 minutes at the room temperature. The sections were cut and stained with HE (haematoxylin and eosin), Masson's trichrome and Periodic Schiff-Methenamine (PASM) staining. The samples were then observed under an Olympus light microscope (Olympus, Tokyo, Japan)[24].

Cellular culture and treatment

Human renal mesangial cells (HRMCs) were purchased from the American Type Culture Collection (ATCC® CRL-1927™). In the present study, HRMCs were cultured in 5.5 mmol/L standard glucose medium (control group)[28]. The *in vitro* hyperglycaemia injury model was achieved via 25 mmol/L high glucose medium for 12 hours. To activate and/or inhibit the mitochondrial fragmentation, cells were pretreated for 30 minutes with FCCP (5 μM, Sigma-Aldrich, USA) and Mdivi1 (10 mM, Sigma-Aldrich, USA), respectively. To suppress and activate the JNK pathway, SP600125 (SP, 10 μM, Selleck Chemicals) and Anisomycin (Ani, 10 μM, Selleck Chemicals), respectively, were used 2 h before high-glucose stress.

Cellular viability and apoptosis evaluation

MTT was used to analyze the cellular viability. HRMCs ($1x10^6$ cells/well) were cultured on a 96-well plate at 37°C with 5% CO_2. Then, 40 μl of MTT solution (2 mg/ml; Sigma-Aldrich) was added to the medium for 4 h at 37°C with 5% CO_2. Subsequently, the cell medium was discarded, and 80 μl of DMSO was added to the wells for 1 h at 37°C with 5% CO_2 in the dark. The OD of each well was observed at A490 nm via a spectrophotometer (Epoch 2; BioTek Instruments, Inc., Winooski, VT, USA). The LDH release assay was performed according to the manufacturer's instructions [29].

Apoptotic cells were also detected with an In Situ Cell Death Detection Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalog No. C1024) according to the manufacturer's protocol. Briefly, cells were fixed with 4% paraformaldehyde at 37°C for 15 min. Blocking buffer was added to the wells, and then cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. The cells were incubated with TUNEL reaction mixture for 1 h at 37°C. DAPI (Sigma-Aldrich, St. Louis, MO, USA) was used to counterstain the nuclei, and the numbers of TUNEL-positive cells were recorded. Five fields per section and three sections per kidney were examined in each experimental group [30]. To analyze changes in caspase-9, caspase-9 activity kits (Beyotime Institute of Biotechnology, China; Catalog No. C1158) were used according to the manufacturer's protocol. In brief, to measure caspase-9 activity, 5 μl of LEHD-p-NA substrate (4 mM, 200 μM final concentration) was added to the samples for 1 hour at 37°C. Then, the absorbance at 400 nm was recorded via a microplate reader to reflect the caspase-3 and caspase-9 activities. To analyze caspase-3 activity, 5 μL of DEVD-p-NA substrate (4 mM, 200 μM final concentration) was added to the samples for 2 hours at 37°C[31].

Western blotting

After treatment, the infarcted kidneys were collected and washed with ice-cold PBS and lysed with RIPA buffer, and the total protein concentration was measured using the BCA assay (Nanjing Keygen Biotech Co., Ltd., Nanjing, Jiangsu, China). The lysates (50-70 μg) were separated by 10% SDS-polyacrylamide gel (10–15%) electrophoresis (SDS-PAGE)[31]. Proteins were electrotransferred onto the Pure Nitrocellulose Blotting membrane (Life Sciences) (Millipore, Bedford, MA, USA) and then blocked with 5% nonfat milk for 2 hr at room temperature. The primary antibodies used in the present study were as follows: caspase-9 (1:1000, Cell Signaling Technology, #9504), pro-caspase-3 (1:1000, Abcam, #ab13847), cleaved caspase-3 (1:1000, Abcam, #ab49822), Bcl2 (1:1000, Cell Signaling Technology, #3498), c-IAP (1:1000, Cell Signaling Technology, #4952), Bax (1:1000, Cell Signaling Technology, #2772), cyt-c (1:1, 000; Abcam; #ab90529), JNK (1:1, 000; Cell Signaling Technology, #4672), p-JNK (1:1, 000; Cell Signaling Technology, #9251), Drp1 (1:1000, Abcam, #ab56788), Opa1 (1:1000, Abcam, #ab42364), Mfn1 (1:1000, Abcam, #ab57602), Fis1 (1:1000, Abcam, #ab71498), MKP1 (1:1000, Abcam, #ab195261), CaMKII (1:1000, Cell Signaling Technology, #3362), p-CaMKII (1:1000, Cell Signaling Technology, #12716).

Immunofluorescence

The cells were washed twice with PBS, permeabilized in 0.1% Triton X-100 overnight at 4°C. After the fixation procedure, the sections were cryoprotected in a PBS solution supplemented with 0.9 mol/l of sucrose overnight at 4°C. After neutralization with NH4Cl buffer, the sections were permeabilized for 45min with 0.05% saponin/PBS (pH=7.4) and incubated overnight with the following primary antibodie [32]: cyt-c (1:1000; Abcam; #ab90529), caspase-9 (1:1000, Cell Signaling Technology, #9504), cleaved caspase-3 (1:1000, Abcam, #ab49822), Tom20 (mitochondrial marker, 1:1000, Abcam, #ab186735), Fis1 (1:1000, Abcam, #ab71498), and p-CaMKII (1:1000, Cell Signaling Technology, #12716). Mitochondrial fission was evaluated via measuring mitochondria length. Fluorescence intensity was calculated using Image-Pro Plus

6.0 software. Firstly, fluorescence pictures (red and green fluorescence) were converted to the grayscale pictures with the help of Image-Pro Plus 6.0 software. Then, red/green fluorescence intensities were separately recorded as the grayscale intensity. Subsequently, relative grayscale intensity was expressed as a ratio to that of control group.

Mitochondrial function analysis

To observe the mitochondrial potential, JC-1 staining (Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalog No. M34152) was used. Then, 10 mg/ml JC-1 was added to the medium for 10 minutes at 37°C in the dark to label the mitochondria. Normal mitochondrial potential showed red fluorescence, and damaged mitochondrial potential showed green fluorescence [33]. Mitochondrial permeability transition pore (mPTP) opening was evaluated based on a previous study using calcein-AM/cobalt. The relative mPTP opening rate was recorded as the ratio to the control group. Mitochondrial ROS production was analyzed via flow cytometry according to a previous study. Cells were washed with cold PBS and cultured with an ROS probe (1 mg/ml, DHE, Molecular Probes, USA) at 37°C in the dark for 15 minutes. After the cells were washed with cold PBS three times, the cells were collected using 0.25% pancreatin. After resuspension in cold PBS, the cells were analyzed using a flow cytometer (BD FACSVerse; BD Biosciences, San Jose, CA, USA) [34].

Adenovirus overexpression assay

MKP1 gain-of-function assay was achieved via transfecting MKP1 adenovirus. To acquire adenovirus-MKP1 (Ad-MKP1), the adenovirus plasmid was purchased from Vigene Bioscience (Rockville, MD, USA)[18]. Subsequently, 293T cells (purchased from ATCC) were used to generate the Ad-MKP1 using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). The medium supernatant was collected, filtered and amplified to yield Ad-MKP1. Then, Ad-MKP1 was used transfected into HRMCs to induce the MKP1 overexpression assay. The transfection and overexpression efficiency were validated via western blotting.

Statistical analysis

The data in the present study were presented as the means ± SEM of at least 3 independent experiments. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

MKP1 overexpression prevents the development of STZ-mediated diabetes

First, MKP1 expression was analysed using qPCR and western blotting. In the control group, MKP1 was abundant in the kidney tissue at both the transcription (Fig. 1 A) and expression levels (Fig. 1 B-C). Interestingly, in the diabetic kidney, MKP1 transcription and expression were downregulated (Fig. 1 A-C). This finding was further supported *in vitro* using human mesangial cells; MKP1 expression was reduced in response to high-glucose challenge via western blotting (Fig. 1 D-E). This information indicated that chronic hyperglycaemia stress caused MKP1 downregulation in kidney. Subsequently, to observe the functional role of MKP1 in diabetic kidney, MKP1 transgenic (MKP1-TG) mice were used. Then, the biological characterization of MKP1-TG mice in the presence of hyperglycaemic stress was monitored. Body weight, fasting blood glucose, and metabolism parameters were measured. Diabetic mice exhibited increased body weight (Fig. 1 F) and higher levels of blood glucose (Fig. 1 G). Not surprisingly, MKP1 overexpression reduced body weight (Fig. 1 F) and repressed the levels of blood glucose (Fig. 1 G). Moreover, the concentration of HbAc1 (Fig. 1 H) and glucagon (Fig. 1 I) were also increased in diabetic mice but were reversed to near-normal levels with MKP1 overexpression. In addition, diabetic mice had increased systolic pressure when compared to the control group (Fig. 1 J); this alteration was reversed by MKP1 overexpression. Altogether, these data illustrated that chronic hyperglycaemia stress caused MKP1 downregulation, and MKP1 overexpression reduced the development of diabetes.

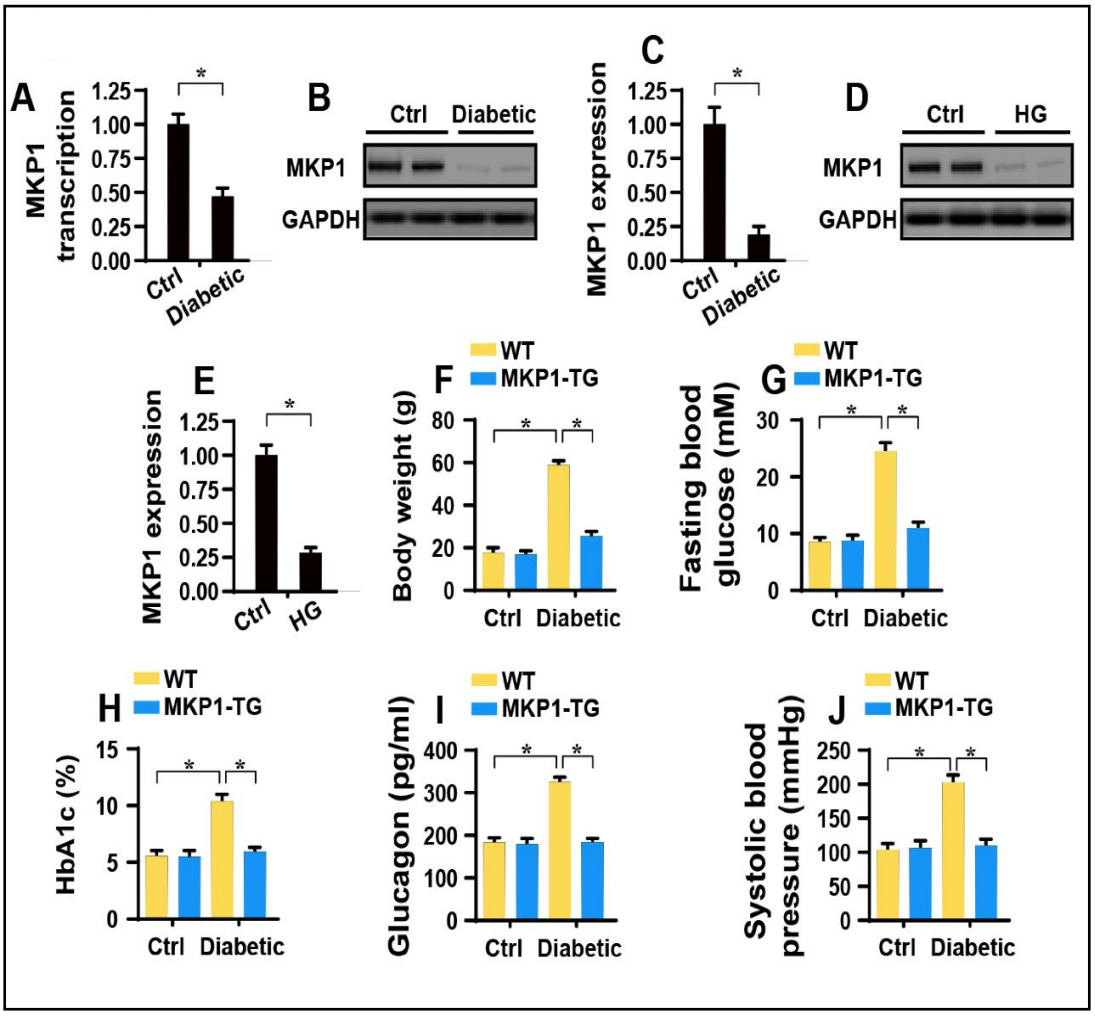

Fig. 1. MKP1 is downregulated in diabetic renal tissue and contributes to the development of diabetes. A. The transcription levels of MKP1 in kidneys from control (ctrl) mice or diabetic mice. B-C. The protein expression of MKP1 in kidneys from control (ctrl) mice or diabetic mice. D-E. In vitro, human mesangial cells were treated with high-glucose (HG) challenge. The protein expression of MKP1 was determined via western blotting. F. Body weight was measured to explore the role of MKP1 in body weight gain. G. Blood glucose levels were measured in WT mice and MKP1-TG mice. H-I. In control mice and MKP1-TG mice, the levels of HbA1c and glucagon were measured via ELISA assay. J. Mouse systolic blood pressures were measured with a CODA semi-automated non-invasive blood pressure device. Experiments were repeated three times, and data are shown as the means ± SEM. n=6 mice per group. *P<0.05.

Reintroduction of MKP1 improves renal function

To gain insight into the beneficial role of MKP1 in diabetic nephropathy, renal function and structure were monitored. As shown in Fig. 2 A-B, elevated BUN and urinary albumin were identified in the diabetic mice, whereas MKP1-TG mice had lower concentrations of BUN and urinary albumin. The albumin/creatinine ratio (ACR) (Fig. 2 C) and serum creatinine (Fig. 2 D) were also increased in response to hyperglycaemia stress and were revered to near-normal levels with MKP1 overexpression. This information identified the protective effects of MKP1 on hyperglycaemia-mediated renal damage.

Functional damage is closely associated with structural alterations. Accordingly, the histopathological changes were assessed. First, the kidney weight was increased in diabetic mice (Fig. 2 E), indicative of renal sclerosis or fibrosis; this effect was negated by MKP1 overexpression. Subsequently, HE staining, PASM staining and Masson's trichrome staining

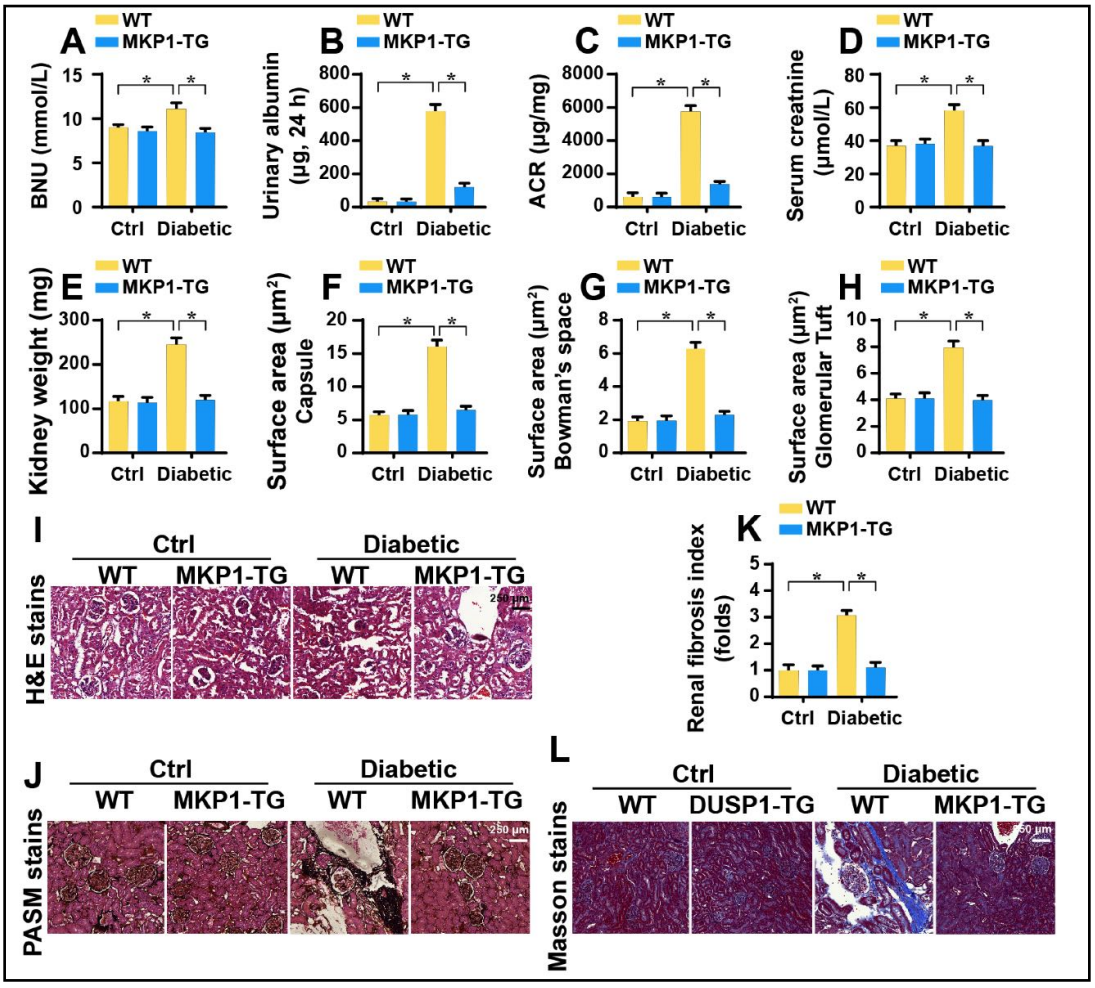

Fig. 2. MKP1 overexpression attenuates renal injury in diabetic mice. A. The blood urea nitrogen (BUN) was measured via ELISA assay in WT mice and MKP1-TG mice. B. To obtain the 24-h urine samples, animals were placed in metabolic cages, and the urinary albumin was measured. C. The urinary albumin content and albumin-creatinine ratio (ACR) were also recorded. D. The blood serum creatinine level was measured via ELISA assay in WT mice and MKP1-TG mice. E. The kidney weight was measured in WT mice and MKP1-TG mice. F-I. The kidney was obtained and the HE staining was performed to observe the structural alterations in diabetic kidneys with MKP1 overexpression. Subsequently, the surface areas in Bowman's capsule, Bowman's space and glomerular tuft were recorded. J. PASM staining of the kidney. K-L. Masson staining for kidneys isolated from WT mice and MKP1-TG mice. The blue area means the renal fibrosis. Experiments were repeated three times, and data are shown as the means ± SEM. n=6 mice per group. *P<0.05.

were performed to observe the glomerulosclerosis, basement membrane thickness and renal fibrosis, respectively. As shown in Fig. 2 F-I, the surface areas in the capsule, Bowman's space and glomerular tuft were all increased in response to chronic hyperglycaemia stress. Interestingly, MKP1 overexpression reversed these histological parameters, suggesting that MKP1 overexpression repressed glomerulosclerosis. Moreover, hyperglycaemia-mediated basement membrane thickness was also attenuated by MKP1 overexpression (Fig. 2 J). These data indicated that MKP1 overexpression sent a beneficial signal for the functional cells in diabetic kidneys. Subsequently, Masson's trichrome staining was used to observe the accumulation of extracellular matrix in the presence of hyperglycaemia stress. Compared to the control group, the fibrosis index was elevated in diabetic kidney and was reduced with MKP1 overexpression (Fig. 2 K-L), suggesting that MKP1 interrupted the collagenous fibre proliferation and thus repressed extracellular matrix deposition.

MKP1 overexpression represses hyperglycaemia-mediated renal oxidative stress and the inflammation response

Oxidative stress and the inflammation response are thought to be the primary reasons for hyperglycaemia-mediated renal function deterioration [35, 36]. Based on this information, experiments were performed to analyse the alteration of oxidative stress markers and the inflammation reaction. First, kidney antioxidant factors such as SOD and GSH-PX were downregulated in diabetic mice and were reversed to near-normal levels in MKP1-TG mice (Fig. 3 A-B). By comparison, oxidative products such as GSSG (oxidized form of GSH) and MDA were increased in diabetic kidneys and were reduced in kidneys with MKP1 overexpression (Fig. 3 C-D). The concentrations of kidney lipid hydroperoxides (LPO) and urinary 8-isoprostane were significantly higher in diabetic mice and were markedly reduced in MKP1-TG mice (Fig. 3 E-F). Altogether, this information indicated that MKP1 overexpression enhanced the antioxidant activity of kidneys in the presence of high-glucose stress.

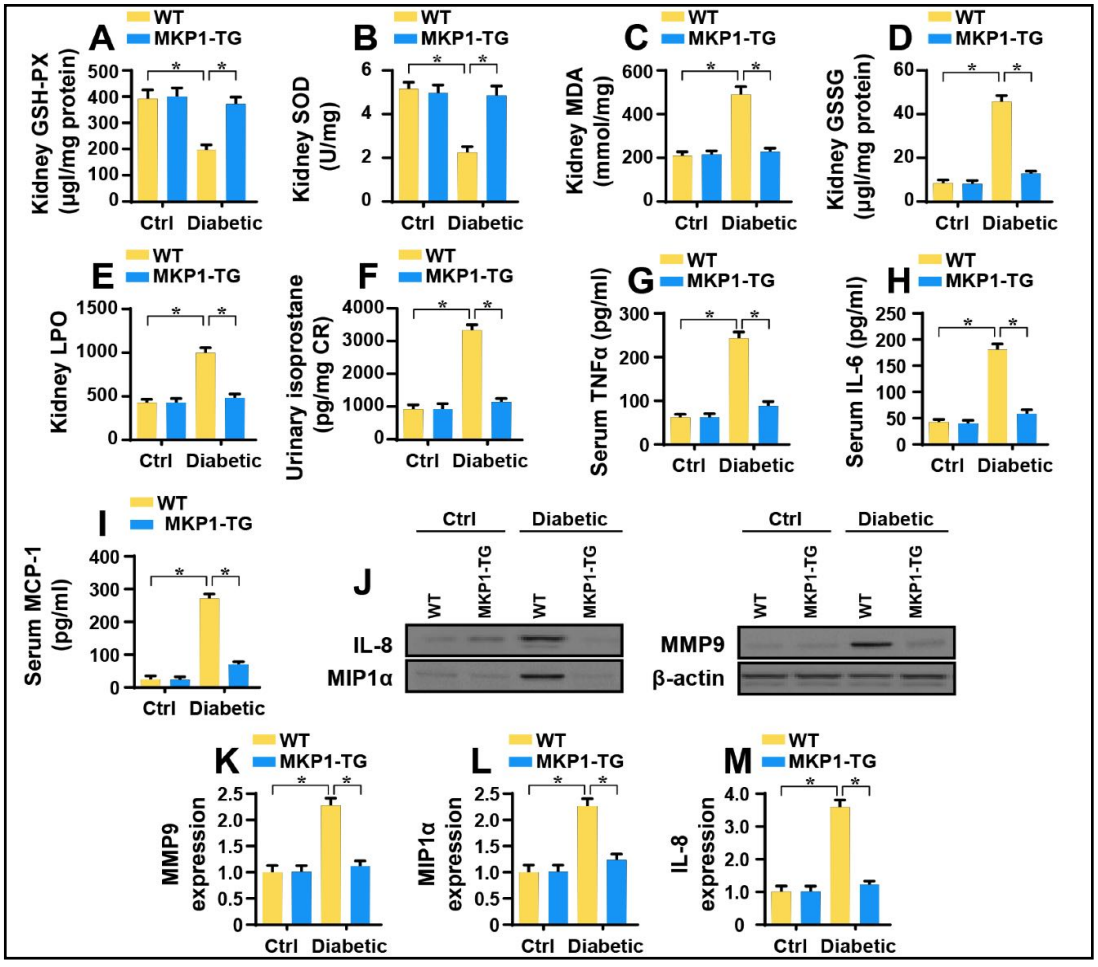

Fig. 3. MKP1 overexpression represses hyperglycaemia-mediated renal oxidative stress and the inflammation response. A-D. Kidneys were collected and the proteins were isolated to analyse the oxidative stress markers via ELISA assay. E-F. The lipid hydroperoxides (LPOs) in kidney homogenates were determined using an LPO kidney assay kit. The urinary 8-isoprostane was measured via collecting the urine in WT mice and MKP1-TG mice. G-I. Blood was collected from WT mice and MKP1-TG mice; then, serum TNFα, IL-6, and MCP-1 were measured via ELISA assay. J-M. Western blotting was performed to analyse the protein expression of inflammation factors. Experiments were repeated three times and data are shown as the means ± SEM. n=6 mice per group. *P<0.05.

With respect to inflammation, ELISA assays demonstrated that the serum TNFα, IL-6 and MCP1 were all increased in the diabetic mice, and this tendency was reversed by MKP1 overexpression (Fig. 3 G-I). Moreover, western blotting using kidney tissues illustrated that MMP9, IL-8 and MIP1α were elevated in response to hyperglycaemia treatment and were reduced to near-normal levels with MKP1 overexpression (Fig. 3 J-M). Altogether, these findings supported the functional importance of MKP1 in suppressing hyperglycaemia-evoked oxidative stress and the inflammation response.

MKP1 reduces renal mesangial cell apoptosis

The next experiments were performed to observe hyperglycaemia-mediated mesangial cell apoptosis [37]. *In vivo*, immunofluorescence assays using the caspase-3 and caspase-9 antibodies demonstrated that hyperglycaemia increased the fluorescence intensity of caspase-3/9 in the glomerulus (Fig. 4 A-B), and this effect was reversed by MKP1 overexpression, indicative of the anti-apoptotic property of MKP1 in diabetic kidney. This finding was further supported by western blotting. As shown in Fig. 4 C-H, hyperglycaemia treatment elevated the expression of pro-apoptotic proteins such as Bax, caspase-3 and caspase-9. In contrast, the expression of anti-apoptotic factors including Bcl-2 and c-IAP was downregulated in diabetic mice. Interestingly, MKP1 overexpression could repress the anti-apoptotic protein upregulation and reverse the content of anti-apoptotic factors (Fig. 4 C-H).

In vitro, the renal mesangial cell was cultured in high-glucose medium, and the apoptotic rate was evaluated via TUNEL assay [38]. Meanwhile, the MKP1 overexpression assay was achieved via transfecting Ad-MKP1 into renal mesangial cell. In the TUNEL assay, we found that the number of TUNEL-positive cells was drastically increased in response to high-glucose treatment (Fig. 4 I-J). Interestingly, MKP1 transfection strongly repressed hyperglycaemia-mediated cell apoptosis (Fig. 4 I-J). This finding was further validated via analysing the cellular viability using MTT assay. As shown in Fig. 4 K, the hyperglycaemia-inhibited cell viability was reversed by MKP1 overexpression. Altogether, our data underscored a pro-survival role played by MKP1 in diabetic kidney *in vitro* and *in vivo*.

MKP1 overexpression attenuates mitochondrial fragmentation via regulating the balance between mitochondrial fission and fusion

Mitochondrial damage has been reported to be the potential target of hyperglycaemia, and fragmented mitochondria are also considered as the initial signal for mesangial cell apoptosis [39, 40]. Given that no study has established the relationship between MKP1 and mitochondrial fragmentation in the presence of hyperglycaemia stress, it is worth investigating whether MKP1 prevented renal dysfunction via repressing the formation of mitochondrial fragmentation. To address this question, immunofluorescence assay for mitochondria was carried out to quantify the mitochondrial fragmentation. As shown in Fig. 5 A, mitochondria in the control group exhibited a highly interconnected phenotype, whereas hyperglycaemia-challenged mitochondria presented a fragmented phenotype. Interestingly, MKP1 overexpression sustained the mitochondrial network and therefore repressed the formation of mitochondrial debris (Fig. 5 A). Subsequently, the average mitochondrial length was measured, and the results illuminated that shorter mitochondria appeared in response to hyperglycaemia treatment (Fig. 5 B). However, mitochondria were longer in the MKP1-overexpressing cells than in the hyperglycaemia group (Fig. 5 B). This finding confirmed that mitochondrial fragmentation was triggered by hyperglycaemia and was inhibited by MKP1 overexpression. Mitochondrial fragmentation is the consequence of excessive mitochondrial fission and/or impaired mitochondrial fusion [15, 19]. Western blotting analysis demonstrated that mitochondrial fission-related proteins such as Drp1 and Fis1 were significantly upregulated in hyperglycaemia-treated cells (Fig. 5 C-G). By comparison, mitochondrial fusion-associated factors such as Opa1 and Mfn1 were correspondingly downregulated under high-glucose treatment (Fig. 5 C-G). Notably, transfection of Ad-MKP1 has the ability to repress the levels of fission-related proteins and reverse the contents of fusion-associated factors. Together, these results revealed the existence of mitochondrial fragmentation in chronic hyperglycaemia-treated kidneys due to the MKP1 deficiency.

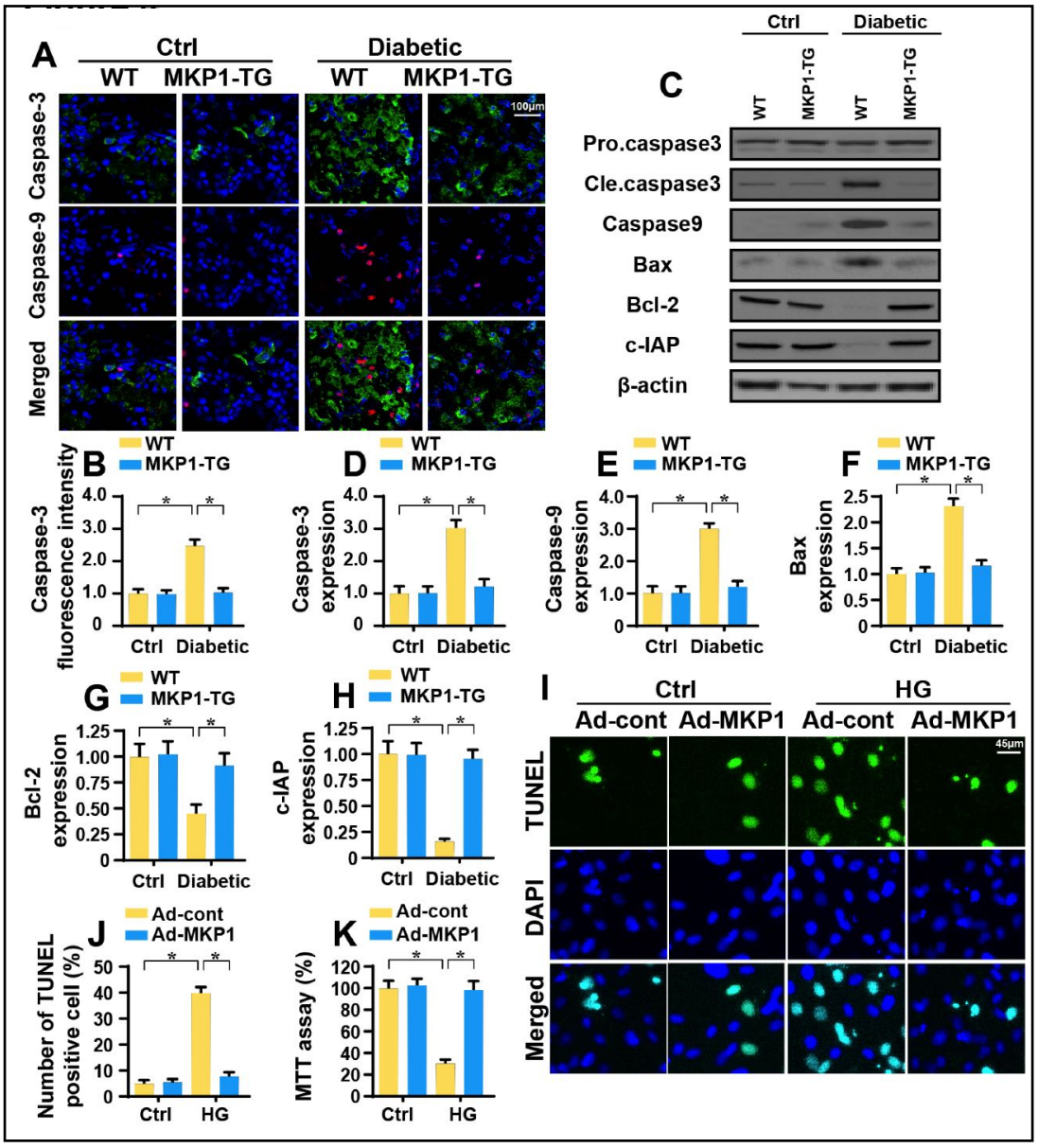

Fig. 4. MKP1 overexpression reduces high glucose-induced glomerular apoptosis. A-B. Immunofluorescence assay for caaspase-3 and caspase-9. The fluorescence intensity of caspase-3 and caspase-9 were significantly increased in response to hyperglycemia and was reduced by MKP1 overexpression. C-H. Proteins were isolated in WT mice and MKP1-TG mice; then, western blotting was performed to analyse the apoptotic protein expression. I-J. In vitro, human mesangial cells were treated with HG stress and then, TUNEL staining was used to quantify the apoptotic cell. K. The renal mesangial cells were cultured under normal-glucose medium (5.5 mmol/L) and high-glucose medium (25 mmol/L) for 12 hours. To reverse the MKP1 overexpression, control adenovirus (Ad-ctrl) and MKP1-adenovirus (Ad-MKP1) were transfected into cells before high-glucose stimulus. Human mesangial cells viability was measured via MTT assay. Ad-MKP1 was transfected into human mesangial cells. Experiments were repeated three times and data are shown as the means ± SEM. n=6 mice per group. Ad-ctrl: control adenovirus; Ad-MKP1: MKP1 adenovirus; HG: high glucose. *P<0.05. *P<0.05.

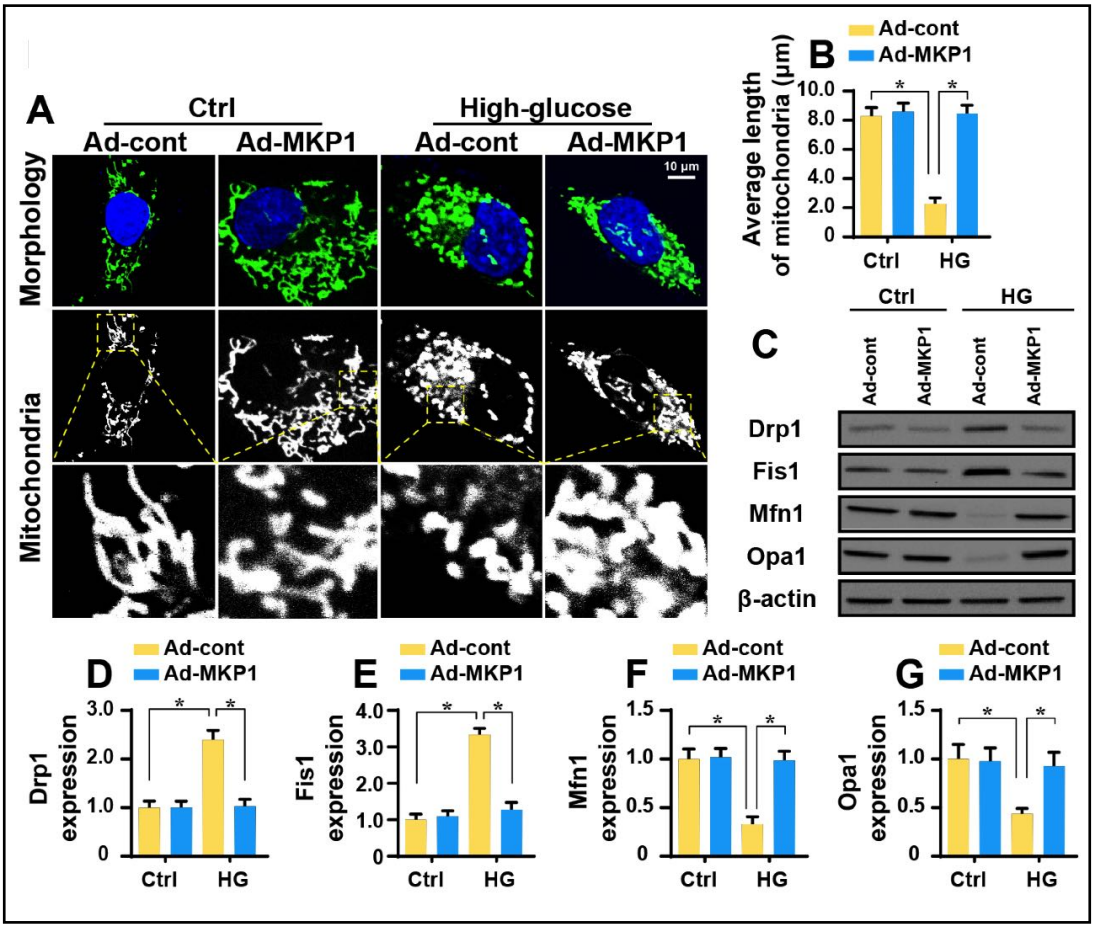

Fig. 5. MKP1 suppresses high-glucose-mediated mitochondrial fragmentation. A-B. The renal mesangial cells were cultured under normal-glucose medium (5.5 mmol/L) and high-glucose medium (25 mmol/L) for 12 hours. To reverse the MKP1 overexpression, control adenovirus (Ad-ctrl) and MKP1-adenovirus (Ad-MKP1) were transfected into cells before high-glucose stimulus. After high-glucose treatment, mitochondria were observed via immunofluorescence assay. Then, the average length of the mitochondria was recorded. C-G. To quantify mitochondrial fission, proteins were isolated and Western blotting was performed to analyse the expression of proteins related to mitochondrial fission/fusion. Experiments were repeated three times, and data are shown as the means ± SEM. Ad-ctrl: control adenovirus; Ad-MKP1: MKP1 adenovirus; HG: high glucose. *P<0.05.

Mitochondrial fragmentation accounts for hyperglycaemia-mediated mitochondrial dysfunction.

Subsequently, we investigated mitochondrial fragmentation in hyperglycaemia-treated renal mesangial cells. To answer this question, we focused on the alterations of mitochondrial function and structure, which are the potential target of high-glucose stress. Mitochondrial potential was reduced in the presence of high-glucose treatment and was reversed to near normal levels with MKP1 overexpression (Fig. 6 A-B). To explain the action of mitochondrial fragmentation, its inhibitor and activator were used. Mdivi-1, the inhibitor of mitochondrial fragmentation, was applied to hyperglycaemia-treated cells, which was used as the negative control group [41]. In MKP1-overexpressing cells, FCCP, an activator of mitochondrial fragmentation, was administered, which was used as the positive control group. After supplementation of Mdivi-1, hyperglycaemia-repressed mitochondrial potential was reversed, similar to the results obtained via overexpression of MKP1 (Fig. 6 A-B). Interestingly, MKP1-stabilized mitochondrial potential was reduced upon activation of mitochondrial fragmentation via FCCP (Fig. 6 A-B). In addition, mitochondrial ROS

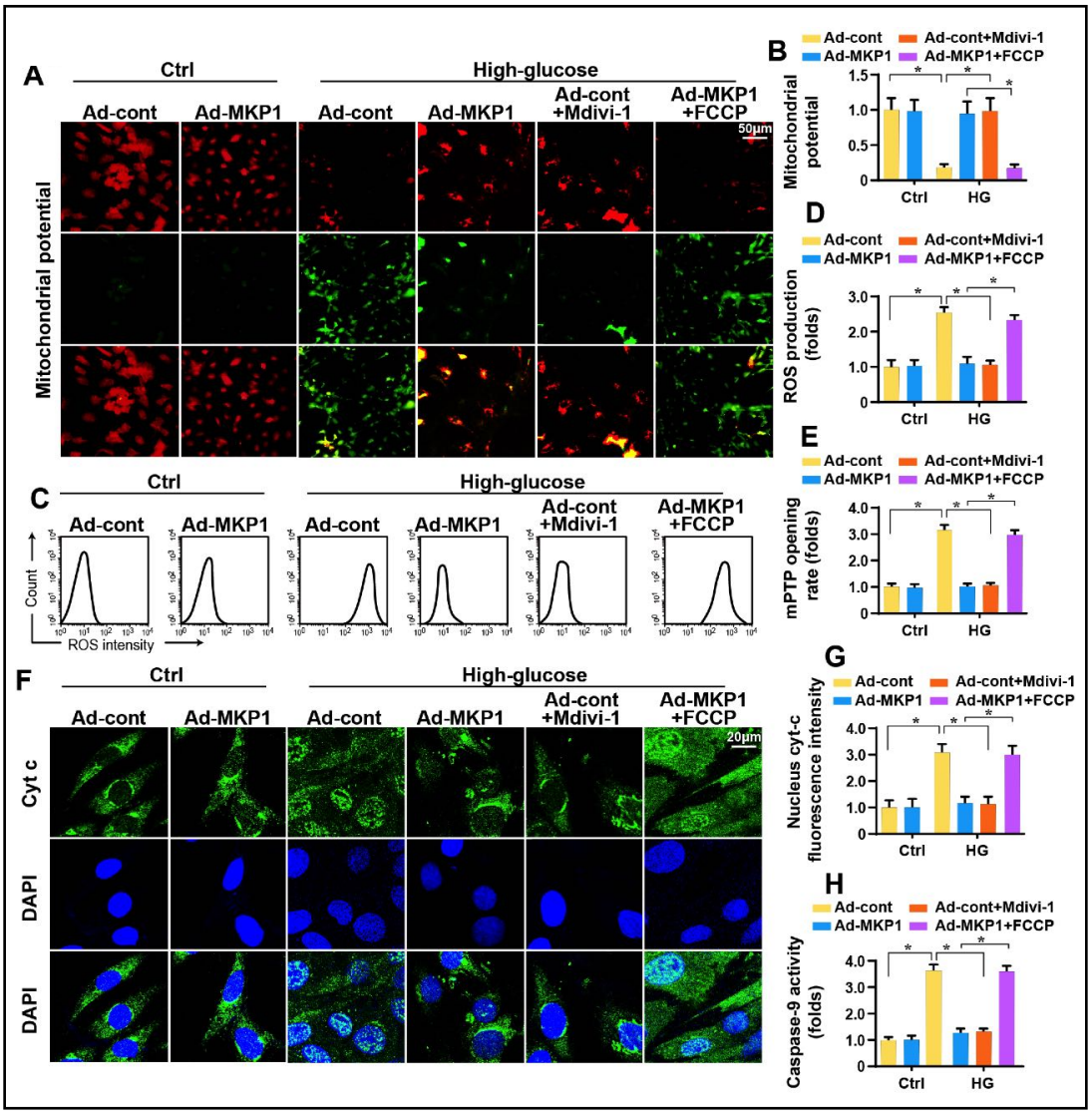

Fig. 6. MKP1-related mitochondrial fragmentation modulates mitochondrial function. A-B. The renal mesangial cells were cultured under normal-glucose medium (5.5 mmol/L) and high-glucose medium (25 mmol/L) for 12 hours. To reverse the MKP1 overexpression, control adenovirus (Ad-ctrl) and MKP1-adenovirus (Ad-MKP1) were transfected into cells before high-glucose stimulus. Mitochondrial membrane potential was observed via JC-1 staining. Red fluorescence indicates the normal mitochondrial potential, whereas green fluorescence indicates the damaged mitochondrial potential. To verify the role of mitochondrial fission in cellular apoptosis, loss- and gain-of-function assays of mitochondrial fission was performed. FCCP, an activator of mitochondrial fission, was added to MKP1-overexpressing cells to re-activate mitochondrial fission. Mdivi-1, the inhibitor of mitochondrial fission, was administered to HG-treated cells to suppress mitochondrial fission. C-D. After high-glucose treatment, ROS staining was performed using an ROS probe. The relative ROS production was measured. E. mPTP opening was recorded with or without DUSP1 overexpression. F-G. Immunofluorescence assay for cyt-c. In response to high-glucose treatment, more cyt-c was released into the nucleus, and this effect was inhibited by MKP1 overexpression. H. Subsequently, caspase-9 activity was measured. Experiments were repeated three times, and data are shown as the means ± SEM. Ad-ctrl: control adenovirus; Ad-MKP1: MKP1 adenovirus; HG: high glucose. *P<0.05.

production was also regulated by MKP1 via inhibiting mitochondrial fragmentation in the presence of hyperglycaemia stress (Fig. 6 C-D). Similarly, the mitochondrial permeability transition pore opening (mPTP) rate was repressed by MKP1 in a mitochondrial fragmentation-dependent manner (Fig. 6 E). Damaged mitochondria would release cyt-c into the cytoplasm/nucleus (Fig. 6 F-G), initiating caspase-9-related mitochondrial apoptosis [42, 43]. Through an immunofluorescence assay for cyt-c, we found that hyperglycaemia mediated more cyt-c leakage into the cytoplasm/nucleus; this effect was negated by MKP1 overexpression via modulating mitochondrial fragmentation. As a consequence of cyt-c liberation, caspase-9 activity was augmented by hyperglycaemia and inhibited by MKP1 overexpression via controlling mitochondrial fragmentation (Fig. 6 H). Altogether, our data confirmed the dispensable role played by mitochondrial fragmentation in hyperglycaemia-related mitochondrial dysfunction. MKP1 overexpression protected mitochondria against hyperglycaemia attack via eliminating mitochondrial fragmentation.

MKP1 controls mitochondrial fragmentation via inhibiting the JNK-CaMKII-Fis1 pathway

Subsequently, we explored the molecular mechanism by which MKP1 inhibited hyperglycaemia-initiated mitochondrial fragmentation. MKP1 is a threonine-tyrosine dual-specificity phosphatase that dephosphorylates and inactivates the MAPK-JNK pathway in kidney. Interestingly, the JNK pathway has been confirmed to be the upstream signal for mitochondrial fragmentation [18]. Other studies also suggest that CaMKII phosphorylation and Fis1 upregulation also participate into the regulation of mitochondrial fragmentation [44, 45]. Based on these findings, we examined whether MKP1 controlled mitochondrial fragmentation via the JNK-CaMKII-Fis1 pathway. The Western blotting analysis shown in Fig. 7 A-D demonstrated that the JNK pathway was activated by hyperglycaemia, as evidenced by increased expression of phosphorylated JNK. Moreover, high-glucose treatment also elevated CaMKII phosphorylation and Fis1 expression (Fig. 7 A-D). Interestingly, MKP1 overexpression repressed JNK activation, CaMKII phosphorylation and Fis1 upregulation (Fig. 7 A-D). To demonstrate whether the JNK pathway is necessary for hyperglycaemia-mediated mitochondrial fragmentation, loss- and gain-of-function assays for the JNK pathway were performed using a pathway blocker and agonist. SP600125 (SP), a JNK pathway blocker, was added to hyperglycaemia-treated cells [46], which was used as the negative control group. Meanwhile, Anisomycin (Ani), a JNK pathway agonist, was administered to MKP1-overexpressing cells, which was used as the positive control group [47]. After blockade of JNK pathway in hyperglycaemia-treated cells, JNK phosphorylation was downregulated, accompanied with a drop in CaMKII phosphorylation and Fis1 expression (Fig. 7 A-D), similar to the results obtained via overexpressing MKP1. However, the inhibitory effects of MKP1 on the JNK-CaMKII-Fis1 pathway were nullified by Ani (Fig. 7 A-D). These data established the regulatory action of MKP1 on the JNK-CaMKII-Fis1 pathway. This finding was further validated via immunofluorescence. Hyperglycaemic stress elevated the fluorescence intensity of p-CaMKII and Fis1 (Fig. 7 E-G); these effects were abrogated by MKP1 overexpression via modulating JNK activity.

To directly observe the mitochondrial fragmentation, an immunofluorescence assay for mitochondria was conducted. As shown in Fig. 7 H, hyperglycaemia-evoked mitochondrial debris was attenuated by MKP1 overexpression or JNK inhibition. Interestingly, Ani treatment abrogated the inhibitory effect of MKP1 on mitochondrial fragmentation. This finding was further supported via measuring the average mitochondrial length (Fig. 7 I). Altogether, our data highlighted that MKP1 regulated mitochondrial fragmentation via the JNK-CaMKII-Fis1 pathway in the presence of high-glucose treatment.

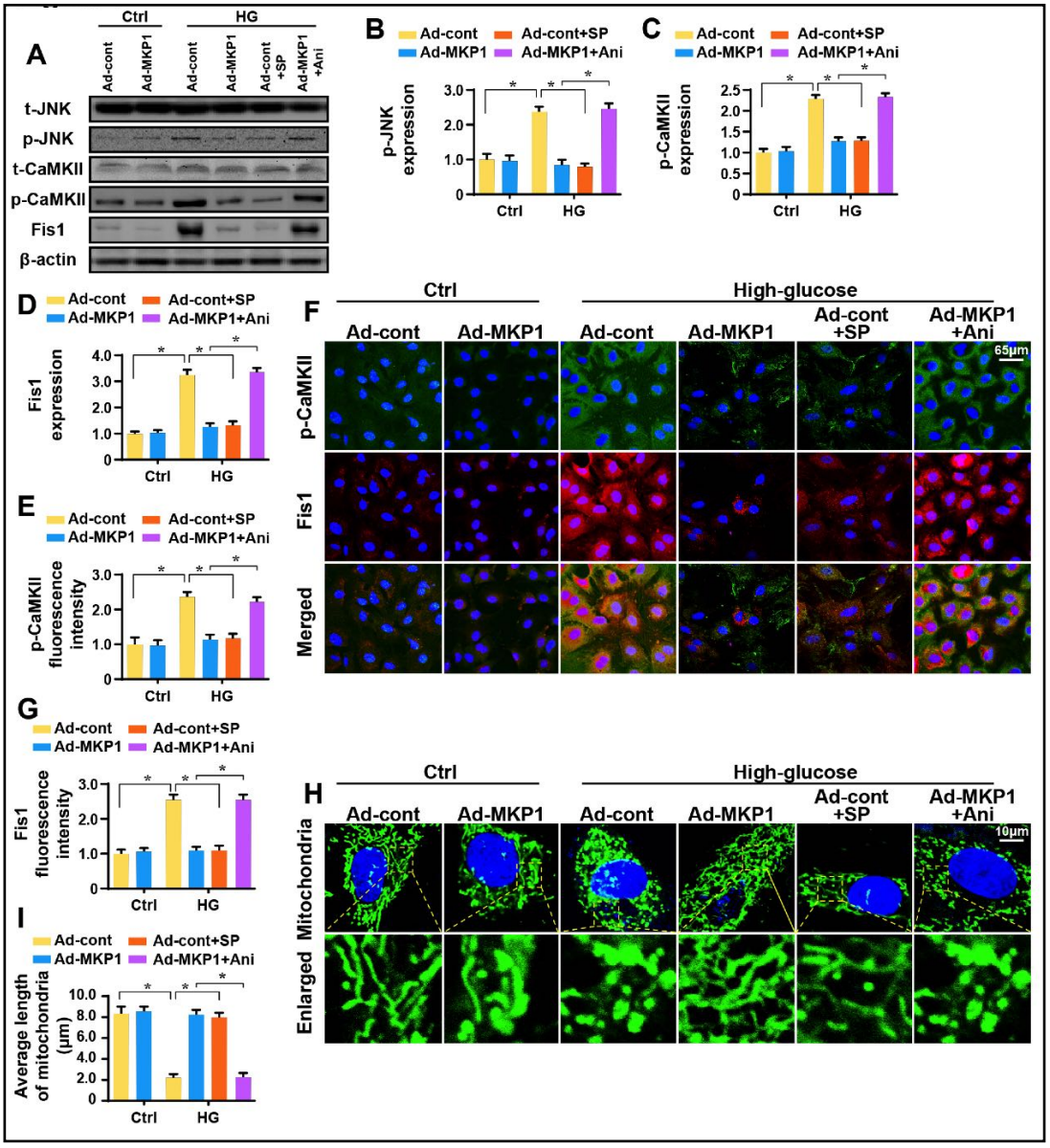

Fig. 7. MKP1 regulates mitochondrial fragmentation via JNK-CaMKII-Fis1 pathways. A-D. After high glucose treatment, proteins were isolated and western blotting was performed to analyse the expression of p-JNK, p-CaMKII and Fis1. Loss- and gain-of-function assays for the JNK pathway were performed using a pathway blocker and agonist. SP600125 (SP), a JNK pathway blocker, was added to hyperglycaemia-treated cells, which was used as the negative control group. Meanwhile, Anisomycin (Ani), a JNK pathway agonist, was administered to MKP1-overexpressing cells, which was used as the positive control group. E-G. Immunofluorescence assay for Fis1 and p-CaMKII in response to MKP1 overexpression and JNK activation in the presence of hyperglycaemia. H-I. Mitochondrial fission was observed via immunofluorescence and the average length of mitochondria was measured. Experiments were repeated three times, and data are shown as the means ± SEM. Ad-ctrl: control adenovirus; Ad-MKP1: MKP1 adenovirus; HG: high glucose. *P<0.05.

JNK-CaMKII-Fis1 pathway is also involved in hyperglycaemia-mediated mesangial cell damage

We explored whether the JNK-CaMKII-Fis1 pathway played a role in mesangial cell apoptosis and mitochondrial dysfunction. As shown in Fig. 8 A, cell death, as assessed via LDH release assay, was augmented by hyperglycaemia and was inhibited by MKP1 overexpression via modifying the JNK-CaMKII-Fis1 pathway [48, 49]. Similarly, hyperglycaemia-activated caspase-3 was repressed by MKP1 overexpression or JNK inhibition (Fig. 8 B). This information confirmed our hypothesis that the JNK-CaMKII-Fis1 pathway was involved in mesangial cell apoptosis in the setting of hyperglycaemic stress. Regarding mitochondrial function, cyt-c liberation, ATP production and redox balance were evaluated. First, hyperglycaemia-mediated cyt-c liberation was repressed by MKP1 overexpression in a manner dependent upon the JNK-CaMKII-Fis1 pathway (Fig. 8 C-E). Similarly, ATP production was reduced by hyperglycaemia and was increased in MKP1-overexpressed cells via modulating the JNK-CaMKII-Fis1 pathway (Fig. 8 F). Finally, the concentration of cellular antioxidants such as SOD, GSH and GPX decreased in response to hyperglycaemia treatment and was reversed to normal levels by MKP1 overexpression in a manner dependent upon the JNK-CaMKII-Fis1 pathway (Fig. 8 G-I). Taken together, our data illustrated that the JNK-CaMKII-Fis1 pathway was also involved in mesangial cell apoptosis and mitochondrial dysfunction in the context of hyperglycaemic challenge.

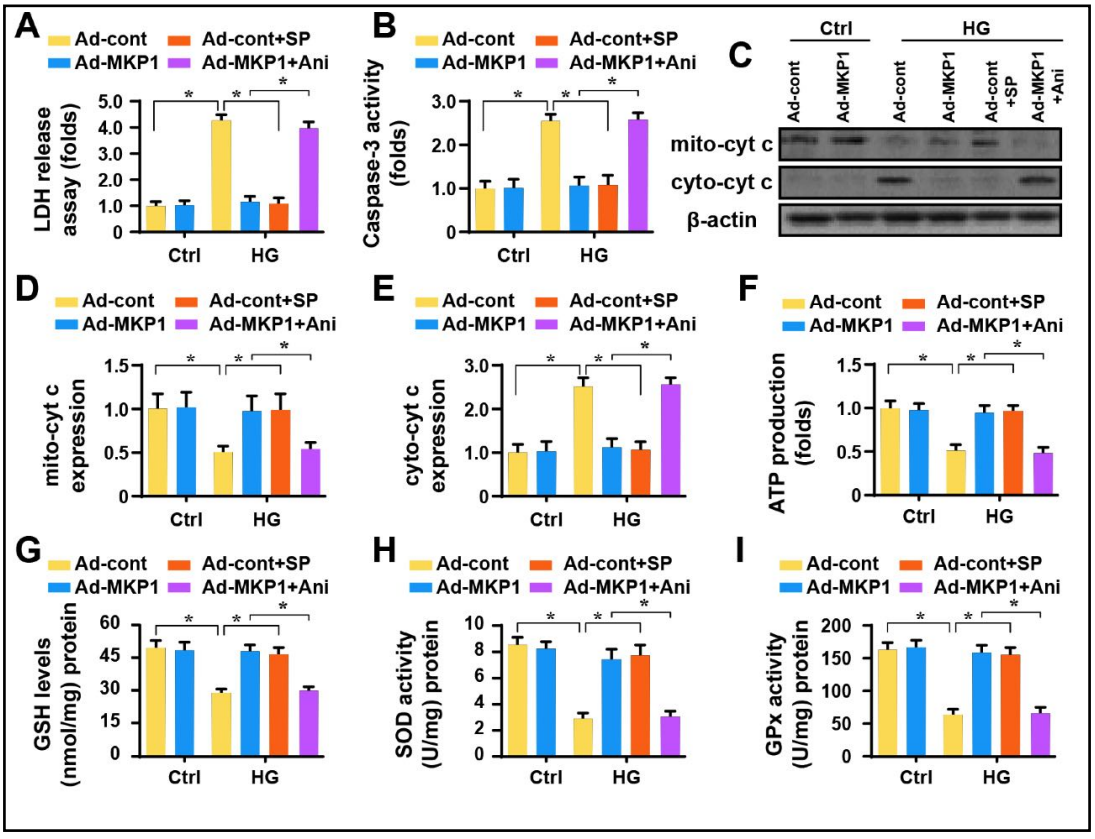

Fig. 8. JNK-CaMKII-Fis1 axis is also involved in glomerular survival. A. After high-glucose treatment, LDH release assay was used to verify the role of JNK-CaMKII-Fis1 axis in glomerular survival. B. Caspase-3 activity assay in response to MKP1 overexpression and JNK activation in the presence of hyperglycaemia. C-E. After high-glucose treatment, proteins were isolated and Western blotting was performed to analyse the expression of cyt-c sub-cellular location. F. ATP production in MKP1-overexpressed cells with JNK activation. G-I. The concentration of cellular antioxidant in MKP1-overexpressed cells with JNK activation. Experiments were repeated three times, and data are shown as the means ± SEM. Ad-ctrl: control adenovirus; Ad-MKP1: MKP1 adenovirus; HG: high glucose. *P<0.05.

Discussion

Diabetic nephropathy, a leading cause of end-stage renal disease, accounts for the chronic drop in renal function. Currently, it has been proposed that 40% of diabetes patients may develop diabetic nephropathy [50]. The primary pathogenesis underlying diabetic nephropathy is the loss of functional cells and the accumulation of extracellular matrix (ECM). Excessive apoptosis of mesangial cells induces a fall in glomerular filtration, along with the thickness of the basement membrane and renal fibrosis [51]. The clinical outcome of diabetic nephropathy is urotoxin retention and metabolic substance accumulation [52]. Therefore, it is necessary to understand the molecular features of hyperglycaemia-mediated renal injury. In the present study, our data elucidated that MKP1 could be considered a potential target to protect kidneys against hyperglycaemic attack. Functional studies demonstrated that MKP1 was downregulated in response to chronic high-glucose stress. However, MKP1 overexpression improved the metabolic parameters, enhanced glucose control, sustained renal function, attenuated kidney oxidative stress, inhibited the renal inflammation response, alleviated mesangial cell apoptosis, and repressed tubulointerstitial fibrosis. Molecular investigation found that MKP1 overexpression enhanced the resistance of mesangial cells to the hyperglycaemic injury via modulating mitochondrial fragmentation. Hyperglycaemia-triggered mitochondrial fragmentation due to mitochondrial dynamic imbalance promoted mitochondrial dysfunction, and this effect was negated by MKP1 via inhibiting the JNK-CaMKII-Fis1 pathway. To the best of our knowledge, this is the first study to describe the comprehensive role of MKP1 in hyperglycaemia-mediated renal injury via *in vivo* and in [53]vitro studies. Our findings provide a potential target to prevent renal dysfunction in patients with diabetic nephropathy. However, more clinical evidence is required in the future to support this concept.

The MKP protein family comprises 11 members and three classes that can dephosphorylate both the serine/threonine and tyrosine residues of their substrates, particularly JNK. Although it has been over a decade since the discovery of MKP1, its precise functions in kidney have not yet been determined. An early study demonstrates that MKP1 plays a key role in regulating nephrogenesis via modification of the JNK pathway. A subsequent study shows that MKP1 deficiency induces JNK activation and thus causes tubular cell damage in response to renal oxidative stress [20]. Furthermore, decreased MKP1 expression is associated with poor prognosis in clear cell renal cell carcinoma [54]. The above information suggests that MKP1 could be considered a defender to sustain kidney homeostasis ranging from renal development and stress response, to tumour formation. In the present study, we found that MKP1 sent a beneficial signal for diabetic kidneys. Downregulated MKP1 resulting from chronic high-glucose stress promoted renal dysfunction and mesangial cell apoptosis. In contrast, MKP1-overexpressing mice are less susceptible to hyperglycaemic damage. These findings, together with the previous reports, have substantiated the sufficiency and necessity of MKP1 in protecting kidney against chronic metabolic injury.

We found that MKP1 overexpression maintained mitochondrial homeostasis. Hyperglycaemia treatment mediated mitochondrial potential loss, redox imbalance, pro-apoptotic factor leakage, and caspase family activation; these effects were strongly rescued by MKP1 overexpression. This finding is in accordance with previous reports. Under chronic high-glucose stress, mitochondria produce excessive ROS that shapes renal oxidative stress. Moreover, structurally poor mitochondria fail to synthesize enough energy to ensure renal functions such as glomerular filtration, tubular resorption and secretion [55]. Moreover, damaged mitochondria cannot be timely removed by autophagy, leading to the leakage of pro-apoptotic factors from mitochondria into the cytoplasm/nucleus [56, 57]. Consequently, extensive cell apoptosis occurs in a mitochondria-dependent manner [58]. Interestingly, our study further identified that mitochondrial dysfunction was primarily attributable to the formation of mitochondrial fragmentation. Due to increased mitochondrial fission and/or decreased mitochondrial fusion, mitochondria mostly divide into non-functional fragments with reduced potential. This mitochondrial debris may be the initial signal for apoptosis

activation because inhibition of mitochondrial fragmentation could terminate caspase-9 activation and reverse mesangial cell viability. These conclusions identify mitochondrial fragmentation as the upstream mediator and trigger event for mitochondrial damage. Our finding is similar to previous studies showing that mitochondrial fragmentation is the pathogenesis for fatty liver [59], gastric cancer [60], cardiac ischaemia-reperfusion injury [15] and diabetic cardiomyopathy [12].

Finally, we found that mitochondrial fragmentation could be repressed by MKP1 via inhibiting the JNK-CaMKII-Fis1 pathway. Actually, previous experiments have identified a causal relationship between JNK activation and excessive mitochondrial fission [18, 61]. In addition, CaMKII phosphorylation has been proved to be the early event regulating the balance between mitochondrial fission and fusion [62]. Besides, Fis1 is the key protein promoting mitochondrial fission in several disease models [63]. In the present study, hyperglycaemia-activated JNK elevated CaMKII phosphorylation and Fis1 expression, accounting for mitochondrial fragmentation. However, MKP1 overexpression interrupted JNK activation and therefore terminated the JNK-CaMKII-Fis1 signalling, favouring mitochondrial homeostasis. This finding is consistent with previous studies [64, 65]. In liver cancer, JNK-CaMKII pathway activation is closely associated with mitochondrial malfunction and cancer migration inhibition [22]. In addition, in the development of atherosclerosis, excessive CaMKII activation and following mitochondrial damage have been reported to be independent risks for atherosclerosis formation [66, 67]. Accordingly, repressing the activity of the JNK-CaMKII-Fis1 pathway is vital to ensure mitochondrial integrity in various disease models.

Conclusion

Collectively, our data confirmed the important role played by MKP1 in diabetic renal injury *in vivo* and *in vitro*. MKP1 deficiency promoted mitochondria damage and mesangial cell apoptosis under high-glucose stress conditions. In this process, mitochondrial fragmentation was the indispensable element in transmitting and amplifying damage signals to initiate mesangial cell death. However, MKP1 overexpression interrupted mitochondrial fragmentation via inhibiting the JNK-CaMKII-Fis1 axis and sustained mitochondrial function, contributing to mesangial cell survival and renal function improvement. These findings suggest that enhancement of MKP1 content in diabetic kidney might be a practical and efficient adjuvant for the treatment of diabetic nephropathy.

Disclosure Statement

The authors declared that they have no conflicts of interest.

References

1 Haneda M, Utsunomiya K, Koya D, Babazono T, Moriya T, Makino H, Kimura K, Suzuki Y, Wada T, Ogawa S, Inaba M, Kanno Y, Shigematsu T, Masakane I, Tsuchiya K, Honda K, Ichikawa K, Shide K, Joint Committee on Diabetic N: A new Classification of Diabetic Nephropathy 2014: a report from Joint Committee on Diabetic Nephropathy. J Diabetes Investig 2015;6:242-246.

2 Zhang J, Zhang Q, Chen X, Liu Y, Xue J, Dahan A, Zhang H, Chai Y: Revealing Synergistic Mechanism of Multiple Components in Gandi Capsule for Diabetic Nephropathy Therapeutics by Network Pharmacology. Evid Based Complement Alternat Med 2018;2018:6503126.

3 Bakris GL, Molitch M: Are All Patients With Type 1 Diabetes Destined for Dialysis if They Live Long Enough? Probably Not. Diabetes Care 2018;41:389-390.

4 Rodriguez-Garcia A, Hevia D, Mayo JC, Gonzalez-Menendez P, Coppo L, Lu J, Holmgren A, Sainz RM: Thioredoxin 1 modulates apoptosis induced by bioactive compounds in prostate cancer cells. Redox Biol 2017;12:634-647.
5 Yu S, Wang X, Geng P, Tang X, Xiang L, Lu X, Li J, Ruan Z, Chen J, Xie G, Wang Z, Ou J, Peng Y, Luo X, Zhang X, Dong Y, Pang X, Miao H, Chen H, Liang H: Melatonin regulates PARP1 to control the senescence-associated secretory phenotype (SASP) in human fetal lung fibroblast cells. J Pineal Res 2017;63:e12405.
6 Czajka A, Malik AN: Hyperglycemia induced damage to mitochondrial respiration in renal mesangial and tubular cells: Implications for diabetic nephropathy. Redox Biol 2016;10:100-107.
7 Shen YQ, Guerra-Librero A, Fernandez-Gil BI, Florido J, Garcia-Lopez S, Martinez-Ruiz L, Mendivil-Perez M, Soto-Mercado V, Acuna-Castroviejo D, Ortega-Arellano H, Carriel V, Diaz-Casado ME, Reiter RJ, Rusanova I, Nieto A, Lopez LC, Escames G: Combination of melatonin and rapamycin for head and neck cancer therapy: Suppression of AKT/mTOR pathway activation, and activation of mitophagy and apoptosis via mitochondrial function regulation. J Pineal Res 2018;64:e12461.
8 Liu Z, Gan L, Xu Y, Luo D, Ren Q, Wu S, Sun C: Melatonin alleviates inflammasome-induced pyroptosis through inhibiting NF-kappaB/GSDMD signal in mice adipose tissue. J Pineal Res 2017;63:e12414.
9 Pant K, Yadav AK, Gupta P, Islam R, Saraya A, Venugopal SK: Butyrate induces ROS-mediated apoptosis by modulating miR-22/SIRT-1 pathway in hepatic cancer cells. Redox Biol 2017;12:340-349.
10 Zhou H, Li D, Zhu P, Hu S, Hu N, Ma S, Zhang Y, Han T, Ren J, Cao F, Chen Y: Melatonin suppresses platelet activation and function against cardiac ischemia/reperfusion injury via PPARgamma/FUNDC1/mitophagy pathways. J Pineal Res 2017;63:e12438.
11 Zhou H, Wang S, Hu S, Chen Y, Ren J: ER-Mitochondria Microdomains in Cardiac Ischemia-Reperfusion Injury: A Fresh Perspective. Front Physiol 2018;9:755.
12 Zhou H, Wang S, Zhu P, Hu S, Chen Y, Ren J: Empagliflozin rescues diabetic myocardial microvascular injury via AMPK-mediated inhibition of mitochondrial fission. Redox Biol 2018;15:335-346.
13 Tobisawa T, Yano T, Tanno M, Miki T, Kuno A, Kimura Y, Ishikawa S, Kouzu H, Nishizawa K, Yoshida H, Miura T: Insufficient activation of Akt upon reperfusion because of its novel modification by reduced PP2A-B55alpha contributes to enlargement of infarct size by chronic kidney disease. Basic Res Cardiol 2017;112:31.
14 Zhou H, Shi C, Hu S, Zhu H, Ren J, Chen Y: BI1 is associated with microvascular protection in cardiac ischemia reperfusion injury via repressing Syk-Nox2-Drp1-mitochondrial fission pathways. Angiogenesis 2018;21:599-615.
15 Zhou H, Wang J, Zhu P, Zhu H, Toan S, Hu S, Ren J, Chen Y: NR4A1 aggravates the cardiac microvascular ischemia reperfusion injury through suppressing FUNDC1-mediated mitophagy and promoting Mff-required mitochondrial fission by CK2alpha. Basic Res Cardiol 2018;113:23.
16 Vargas LA, Velasquez FC, Alvarez BV: Compensatory role of the NBCn1 sodium/bicarbonate cotransporter on Ca(2+)-induced mitochondrial swelling in hypertrophic hearts. Basic Res Cardiol 2017;112:14.
17 Han H, Cao A, Wang L, Guo H, Zang Y, Li Z, Zhang X, Peng W: Huangqi Decoction Ameliorates Streptozotocin-Induced Rat Diabetic Nephropathy through Antioxidant and Regulation of the TGF-beta/MAPK/PPAR-gamma Signaling. Cell Physiol Biochem 2017;42:1934-1944.
18 Jin Q, Li R, Hu N, Xin T, Zhu P, Hu S, Ma S, Zhu H, Ren J, Zhou H: DUSP1 alleviates cardiac ischemia/reperfusion injury by suppressing the Mff-required mitochondrial fission and Bnip3-related mitophagy via the JNK pathways. Redox Biol 2018;14:576-587.
19 Zhou H, Hu S, Jin Q, Shi C, Zhang Y, Zhu P, Ma Q, Tian F, Chen Y: Mff-Dependent Mitochondrial Fission Contributes to the Pathogenesis of Cardiac Microvasculature Ischemia/Reperfusion Injury via Induction of mROS-Mediated Cardiolipin Oxidation and HK2/VDAC1 Disassociation-Involved mPTP Opening. J Am Heart Assoc 2017;6:e005328.
20 Xu Q, Konta T, Nakayama K, Furusu A, Moreno-Manzano V, Lucio-Cazana J, Ishikawa Y, Fine LG, Yao J, Kitamura M: Cellular defense against H2O2-induced apoptosis via MAP kinase-MKP-1 pathway. Free Radic Biol Med 2004;36:985-993.
21 Zhou H, Zhu P, Guo J, Hu N, Wang S, Li D, Hu S, Ren J, Cao F, Chen Y: Ripk3 induces mitochondrial apoptosis via inhibition of FUNDC1 mitophagy in cardiac IR injury. Redox Biol 2017;13:498-507.
22 Shi C, Cai Y, Li Y, Li Y, Hu N, Ma S, Hu S, Zhu P, Wang W, Zhou H: Yap promotes hepatocellular carcinoma metastasis and mobilization via governing cofilin/F-actin/lamellipodium axis by regulation of JNK/Bnip3/SERCA/CaMKII pathways. Redox Biol 2018;14:59-71.

23 Zhou H, Ma Q, Zhu P, Ren J, Reiter RJ, Chen Y: Protective role of melatonin in cardiac ischemia-reperfusion injury: From pathogenesis to targeted therapy. J Pineal Res 2018;64:e12471.
24 Feng J, Lu C, Dai Q, Sheng J, Xu M: SIRT3 Facilitates Amniotic Fluid Stem Cells to Repair Diabetic Nephropathy Through Protecting Mitochondrial Homeostasis by Modulation of Mitophagy. Cell Physiol Biochem 2018;46:1508-1524.
25 Torres-Estay V, Carreno DV, Fuenzalida P, Watts A, San Francisco IF, Montecinos VP, Sotomayor PC, Ebos J, Smith GJ, Godoy AS: Androgens modulate male-derived endothelial cell homeostasis using androgen receptor-dependent and receptor-independent mechanisms. Angiogenesis 2017;20:25-38.
26 Randriamboavonjy V, Kyselova A, Elgheznawy A, Zukunft S, Wittig I, Fleming I: Calpain 1 cleaves and inactivates prostacyclin synthase in mesenteric arteries from diabetic mice. Basic Res Cardiol 2017;112:10.
27 Zhang Y, Zhou H, Wu W, Shi C, Hu S, Yin T, Ma Q, Han T, Zhang Y, Tian F, Chen Y: Liraglutide protects cardiac microvascular endothelial cells against hypoxia/reoxygenation injury through the suppression of the SR-Ca(2+)-XO-ROS axis via activation of the GLP-1R/PI3K/Akt/survivin pathways. Free Radic Biol Med 2016;95:278-292.
28 Xu J, Wu Y, Lu G, Xie S, Ma Z, Chen Z, Shen HM, Xia D: Importance of ROS-mediated autophagy in determining apoptotic cell death induced by physapubescin B. Redox Biol 2017;12:198-207.
29 Hu SY, Zhang Y, Zhu PJ, Zhou H, Chen YD: Liraglutide directly protects cardiomyocytes against reperfusion injury possibly via modulation of intracellular calcium homeostasis. J Geriatr Cardiol 2017;14:57-66.
30 Murphy PS, Wang J, Bhagwat SP, Munger JC, Janssen WJ, Wright TW, Elliott MR: CD73 regulates anti-inflammatory signaling between apoptotic cells and endotoxin-conditioned tissue macrophages. Cell Death Differ 2017;24:559-570.
31 Sun Y, Li Q, Zhang J, Chen Z, He Q, Liu X, Zhao N, Yin A, Huang H, He M, Cao L, Wang L: Autophagy regulatory molecule, TMEM74, interacts with BIK and inhibits BIK-induced apoptosis. Cell Signal 2017;36:34-41.
32 Das N, Mandala A, Naaz S, Giri S, Jain M, Bandyopadhyay D, Reiter RJ, Roy SS: Melatonin protects against lipid-induced mitochondrial dysfunction in hepatocytes and inhibits stellate cell activation during hepatic fibrosis in mice. J Pineal Res 2017;62:e12404.
33 Gadicherla AK, Wang N, Bulic M, Agullo-Pascual E, Lissoni A, De Smet M, Delmar M, Bultynck G, Krysko DV, Camara A, Schluter KD, Schulz R, Kwok WM, Leybaert L: Mitochondrial Cx43 hemichannels contribute to mitochondrial calcium entry and cell death in the heart. Basic Res Cardiol 2017;112:27.
34 Wang K, Gan TY, Li N, Liu CY, Zhou LY, Gao JN, Chen C, Yan KW, Ponnusamy M, Zhang YH, Li PF: Circular RNA mediates cardiomyocyte death via miRNA-dependent upregulation of MTP18 expression. Cell Death Differ 2017;24:1111-1120.
35 Li R, Xin T, Li D, Wang C, Zhu H, Zhou H: Therapeutic effect of Sirtuin 3 on ameliorating nonalcoholic fatty liver disease: The role of the ERK-CREB pathway and Bnip3-mediated mitophagy. Redox Biol 2018;18:229-243.
36 Jovancevic N, Dendorfer A, Matzkies M, Kovarova M, Heckmann JC, Osterloh M, Boehm M, Weber L, Nguemo F, Semmler J, Hescheler J, Milting H, Schleicher E, Gelis L, Hatt H: Medium-chain fatty acids modulate myocardial function via a cardiac odorant receptor. Basic Res Cardiol 2017;112:13.
37 Alghanem AF, Wilkinson EL, Emmett MS, Aljasir MA, Holmes K, Rothermel BA, Simms VA, Heath VL, Cross MJ: RCAN1.4 regulates VEGFR-2 internalisation, cell polarity and migration in human microvascular endothelial cells. Angiogenesis 2017;20:341-358.
38 Kingery JR, Hamid T, Lewis RK, Ismahil MA, Bansal SS, Rokosh G, Townes TM, Ildstad ST, Jones SP, Prabhu SD: Leukocyte iNOS is required for inflammation and pathological remodeling in ischemic heart failure. Basic Res Cardiol 2017;112:19.
39 Nunez-Gomez E, Pericacho M, Ollauri-Ibanez C, Bernabeu C, Lopez-Novoa JM: The role of endoglin in post-ischemic revascularization. Angiogenesis 2017;20:1-24.
40 Zhou H, Wang J, Zhu P, Hu S, Ren J: Ripk3 regulates cardiac microvascular reperfusion injury: The role of IP3R-dependent calcium overload, XO-mediated oxidative stress and F-action/filopodia-based cellular migration. Cell Signal 2018;45:12-22.
41 Zhou H, Li D, Zhu P, Ma Q, Toan S, Wang J, Hu S, Chen Y, Zhang Y: Inhibitory effect of melatonin on necroptosis via repressing the Ripk3-PGAM5-CypD-mPTP pathway attenuates cardiac microvascular ischemia-reperfusion injury. J Pineal Res 2018;65:e12503.

42 Zhu P, Hu S, Jin Q, Li D, Tian F, Toan S, Li Y, Zhou H, Chen Y: Ripk3 promotes ER stress-induced necroptosis in cardiac IR injury: A mechanism involving calcium overload/XO/ROS/mPTP pathway. Redox Biol 2018;16:157-168.
43 Zhou J, Zhong J, Lin S, Huang Z, Chen H, Tang S, Yang C, Fan Y: Inhibition of PTEN Activity Aggravates Post Renal Fibrosis in Mice with Ischemia Reperfusion-Induced Acute Kidney Injury. Cell Physiol Biochem 2017;43:1841-1854.
44 Li P, Bai Y, Zhao X, Tian T, Tang L, Ru J, An Y, Wang J: NR4A1 contributes to high-fat associated endothelial dysfunction by promoting CaMKII-Parkin-mitophagy pathways. Cell Stress Chaperones 2018;23:749-761.
45 Scarpelli P, Almeida GT, Vicoso KL, Lima WR, Pereira LB, Meissner KA, Wrenger C, Rafaello A, Rizzuto R, Pozzan T, Garcia CRS: Melatonin activate FIS1, DYN1 and DYN2 Plasmodium falciparum related-genes for mitochondria fission: mitoemerald-GFP as a tool to visualize mitochondria structure. J Pineal Res 2018;64:e12484.
46 Jokinen R, Pirnes-Karhu S, Pietilainen KH, Pirinen E: Adipose tissue NAD(+)-homeostasis, sirtuins and poly(ADP-ribose) polymerases -important players in mitochondrial metabolism and metabolic health. Redox Biol 2017;12:246-263.
47 Lee HY, Back K: Melatonin is required for H2 O2 - and NO-mediated defense signaling through MAPKKK3 and OXI1 in Arabidopsis thaliana. J Pineal Res 2017;62:e12379.
48 Kang PT, Chen CL, Lin P, Chilian WM, Chen YR: Impairment of pH gradient and membrane potential mediates redox dysfunction in the mitochondria of the post-ischemic heart. Basic Res Cardiol 2017;112:36.
49 Liu L, Li H, Cui Y, Li R, Meng F, Ye Z, Zhang X: Calcium Channel Opening Rather than the Release of ATP Causes the Apoptosis of Osteoblasts Induced by Overloaded Mechanical Stimulation. Cell Physiol Biochem 2017;42:441-454.
50 Liu D, Zeng X, Li X, Mehta JL, Wang X: Role of NLRP3 inflammasome in the pathogenesis of cardiovascular diseases. Basic Res Cardiol 2017;113:5.
51 Merjaneh M, Langlois A, Larochelle S, Cloutier CB, Ricard-Blum S, Moulin VJ: Pro-angiogenic capacities of microvesicles produced by skin wound myofibroblasts. Angiogenesis 2017;20:385-398.
52 Rossello X, Riquelme JA, He Z, Taferner S, Vanhaesebroeck B, Davidson SM, Yellon DM: The role of PI3Kalpha isoform in cardioprotection. Basic Res Cardiol 2017;112:66.
53 Jahandiez V, Cour M, Bochaton T, Abrial M, Loufouat J, Gharib A, Varennes A, Ovize M, Argaud L: Fast therapeutic hypothermia prevents post-cardiac arrest syndrome through cyclophilin D-mediated mitochondrial permeability transition inhibition. Basic Res Cardiol 2017;112:35.
54 Thirusangu P, Vigneshwaran V, Prashanth T, Vijay Avin BR, Malojirao VH, Rakesh H, Khanum SA, Mahmood R, Prabhakar BT: BP-1T, an antiangiogenic benzophenone-thiazole pharmacophore, counteracts HIF-1 signalling through p53/MDM2-mediated HIF-1alpha proteasomal degradation. Angiogenesis 2017;20:55-71.
55 Sarkar C, Ganju RK, Pompili VJ, Chakroborty D: Enhanced peripheral dopamine impairs post-ischemic healing by suppressing angiotensin receptor type 1 expression in endothelial cells and inhibiting angiogenesis. Angiogenesis 2017;20:97-107.
56 Le Cras TD, Mobberley-Schuman PS, Broering M, Fei L, Trenor CC, 3rd, Adams DM: Angiopoietins as serum biomarkers for lymphatic anomalies. Angiogenesis 2017;20:163-173.
57 Zhou H, Yang J, Xin T, Zhang T, Hu S, Zhou S, Chen G, Chen Y: Exendin-4 enhances the migration of adipose-derived stem cells to neonatal rat ventricular cardiomyocyte-derived conditioned medium via the phosphoinositide 3-kinase/Akt-stromal cell-derived factor-1alpha/CXC chemokine receptor 4 pathway. Mol Med Rep 2015;11:4063-4072.
58 Garcia-Ruiz JM, Galan-Arriola C, Fernandez-Jimenez R, Aguero J, Sanchez-Gonzalez J, Garcia-Alvarez A, Nuno-Ayala M, Dube GP, Zafirelis Z, Lopez-Martin GJ, Bernal JA, Lara-Pezzi E, Fuster V, Ibanez B: Bloodless reperfusion with the oxygen carrier HBOC-201 in acute myocardial infarction: a novel platform for cardioprotective probes delivery. Basic Res Cardiol 2017;112:17.
59 Zhou H, Du W, Li Y, Shi C, Hu N, Ma S, Wang W, Ren J: Effects of melatonin on fatty liver disease: The role of NR4A1/DNA-PKcs/p53 pathway, mitochondrial fission, and mitophagy. J Pineal Res 2018;64:e12450.
60 Hu Z, Cheng J, Xu J, Ruf W, Lockwood CJ: Tissue factor is an angiogenic-specific receptor for factor VII-targeted immunotherapy and photodynamic therapy. Angiogenesis 2017;20:85-96.
61 Wang X, Song Q: Mst1 regulates post-infarction cardiac injury through the JNK-Drp1-mitochondrial fission pathway. Cell Mol Biol Lett 2018;23:21.

62 Zhou H, Zhang Y, Hu S, Shi C, Zhu P, Ma Q, Jin Q, Cao F, Tian F, Chen Y: Melatonin protects cardiac microvasculature against ischemia/reperfusion injury via suppression of mitochondrial fission-VDAC1-HK2-mPTP-mitophagy axis. J Pineal Res 2017;63:e12413.
63 Zhou H, Yue Y, Wang J, Ma Q, Chen Y: Melatonin therapy for diabetic cardiomyopathy: A mechanism involving Syk-mitochondrial complex I-SERCA pathway. Cell Signal 2018;47:88-100.
64 Li H, He F, Zhao X, Zhang Y, Chu X, Hua C, Qu Y, Duan Y, Ming L: YAP Inhibits the Apoptosis and Migration of Human Rectal Cancer Cells via Suppression of JNK-Drp1-Mitochondrial Fission-HtrA2/Omi Pathways. Cell Physiol Biochem 2017;44:2073-2089.
65 Nauta TD, van den Broek M, Gibbs S, van der Pouw-Kraan TC, Oudejans CB, van Hinsbergh VW, Koolwijk P: Identification of HIF-2alpha-regulated genes that play a role in human microvascular endothelial sprouting during prolonged hypoxia *in vitro*. Angiogenesis 2017;20:39-54.
66 Breyer MD, Kretzler M: Novel avenues for drug discovery in diabetic kidney disease. Expert Opin Drug Discov 2018;13:65-74.
67 Dong X, Fu J, Yin X, Qu C, Yang C, He H, Ni J: Induction of Apoptosis in HepaRG Cell Line by Aloe-Emodin through Generation of Reactive Oxygen Species and the Mitochondrial Pathway. Cell Physiol Biochem 2017;42:685-696.