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CKIP-1 participates in the activation of Nrf2 signaling pathway by Cx43 and the regulation of diabetic renal fibrosis

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Abstract

Abstract Background and Purpose We previously reported that both Cx43 and CKIP-1 attenuated diabetic renal fibrosis via the activation of Nrf2 signaling pathway. However, whether CKIP-1, a scaffold protein, participates in regulating the activation of Nrf2 signaling pathway by Cx43 remains to be elucidated. Experimental Approach The effect of adenovirus-mediated Cx43 overexpression on renal fibrosis in CKIP-1-/- diabetic mice was investigated. Cx43 overexpressed plasmid and CKIP-1 small interfering RNA were simultaneously transfected into GMCs and the activity of Nrf2 signaling pathway was observed. The interaction between Cx43 and CKIP-1 was analyzed by immunofluorescence and immunoprecipitation assays. Key Results Overexpression of Cx43 could significantly alleviate renal fibrosis by activating the Nrf2 pathway in diabetic mice, but have no obvious effect in CKIP-1-/- diabetic mice. The effect of activation of Nrf2 signaling pathway by Cx43 was blocked by CKIP-1 depletion. Cx43 interacted with CKIP-1, and the interaction was weakened by high glucose treatment. Cx43 regulated the expression of CKIP-1 and the interaction of CKIP-1 with Nrf2 via Cx43 carboxyl terminus (CT) domain, thereby activating Nrf2 signaling pathway. Conclusion and Implications CKIP-1 participates in regulating the activation of Nrf2 signaling pathway by Cx43, the mechanism of which might be related to the interaction of CKIP-1 with Nrf2 through Cx43 CT. Our study provides further experimental basis for targeting the Cx43-CKIP-1-Nrf2 axis to resist diabetic renal fibrosis.

Background and Purpose

We previously reported that both Cx43 and CKIP-1 attenuated diabetic renal fibrosis via the activation of Nrf2 signaling pathway. However, whether CKIP-1, a scaffold protein, participates in regulating the activation of Nrf2 signaling pathway by Cx43 remains to be elucidated.

Experimental Approach

The effect of adenovirus-mediated Cx43 overexpression on renal fibrosis in *CKIP-1*$^{-/-}$ diabetic mice was investigated. Cx43 overexpressed plasmid and CKIP-1 small interfering RNA were simultaneously transfected into GMCs and the activity of Nrf2 signaling pathway was observed. The interaction between Cx43 and CKIP-1 was analyzed by immunofluorescence and immunoprecipitation assays.

Key Results

Overexpression of Cx43 could significantly alleviate renal fibrosis by activating the Nrf2 pathway in diabetic mice, but have no obvious effect in *CKIP-1*$^{-/-}$ diabetic mice. The effect of activation of Nrf2 signaling pathway by Cx43 was blocked by CKIP-1 depletion. Cx43 interacted with CKIP-1, and the interaction was weakened by high glucose treatment. Cx43 regulated the expression of CKIP-1 and the interaction of CKIP-1 with Nrf2 via Cx43 carboxyl terminus (CT) domain, thereby activating Nrf2 signaling pathway.

Conclusion and Implications

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CKIP-1 participates in regulating the activation of Nrf2 signaling pathway by Cx43, the mechanism of which might be related to the interaction of CKIP-1 with Nrf2 through Cx43 CT. Our study provides further experimental basis for targeting the Cx43-CKIP-1-Nrf2 axis to resist diabetic renal fibrosis.

Key words

Diabetic nephropathy (DN); oxidative stress; Cx43; CKIP-1; Nrf2.

Introduction

Diabetic nephropathy (DN), the main pathological feature of which is renal fibrosis, including glomerulosclerosis and tubulointerstitial fibrosis, is a progressive and long-term diabetic complication with complicated pathogenesis (Gnudi et al., 2016; Kanwar et al., 2011). The increase in renal oxidative stress caused by glucose and lipid metabolism disorders and other factors is one of the main pathogenesis of diabetic renal fibrosis, which has gradually become a consensus (Forbes JM, 2008; Kashihara et al., 2010; Ruiz et al., 2013; Sagoo et al., 2018). The essence of oxidative stress is the disproportionate generation of reactive oxygen species (ROS) and endogenous antioxidants (Brownlee, 2001).*Nuclear factor-erythroid 2-related factor 2 (Nrf2)* is a key transcription factor for cellular antioxidant and cytoprotective genes, which is widely expressed in various tissues and highly expressed in kidney tissues (Cheng et al., 2016; Moi et al., 1994). The activation of Nrf2 anti-oxidative stress signaling pathway could be considered as potential strategies for treating diabetic kidney fibrosis (Choi et al., 2014; Jiang et al., 2010; Zheng et al., 2011). Therefore, further study of associated signaling proteins with the activation of Nrf2 signaling pathway plays a pivotal role in exploring potential targets for resistance to the development of diabetic renal fibrosis.

Connexin 43 (Cx43) is the most prevalent, ubiquitously distributed and by far the best characterized isoform of the gap junction protein family (Bosco et al., 2011; J. Z. Zhou et al., 2014), which accomplishes its biological function not only through gap junction intercellular communication but also through the interaction with a large number of signaling and scaffold proteins via its carboxyl terminus (CT) (Leithe et al., 2018; Ribeiro-Rodrigues et al., 2017). Recently, as Cx43 has been highly valued in the pathological research of diabetic nephropathy (Abed et al., 2014; Hills et al., 2018), related studies on Cx43 as a target to resist diabetic renal fibrosis have gradually become a hot spot. It was revealed that Cx43 was widely distributed in glomerular mesangial cells (GMCs) (Barajas et al., 1994), while the expression of Cx43 was significantly down-regulated in kidney tissues of diabetic patients, diabetic animals and GMCs cultured with high glucose (Liu et al., 2012; Sawai et al., 2006). Our previous study showed that Cx43 could inhibit the up-regulation of fibrosis-related proteins including*fibronectin (FN)* and transforming growth factor-β1 (TGF-β1) in GMCs induced by high glucose by activating the Nrf2 pathway, and improve renal fibrosis (Z. Chen et al., 2017). However, the molecular mechanism of how Cx43 activates the Nrf2 signaling pathway is still unknown.

Casein kinase 2 interacting protein 1 (CKIP-1), a scaffold adaptor, mediating numerous protein–protein interactions through different domains (Bosc et al., 2000), plays an important role in the signal transduction between the cytoplasm and the nucleus which depends on its subcellular localization to mediate the subcellular localization of other proteins and regulate the function of other proteins (Nie et al., 2013). Studies have confirmed the function of CKIP-1 in regulating kinase metabolism disorders and inhibiting inflammatory proliferation response (Y. Chen et al., 2017; Zhan et al., 2017; Luo Zhang et al., 2014). Our previous research found that CKIP-1 might reduce the expression of inflammatory fibrosis factors such as FN and ICAM-1 in diabetic kidneys by activating the Nrf2 signaling pathway, and inhibit the pathological progress of diabetic renal fibrosis (W. Gong et al., 2016). Based on our previous results that Cx43 and CKIP-1 regulating diabetic renal fibrosis were both associated with the activation of Nrf2 anti-oxidative stress signaling pathway, we aimed to investigate whether CKIP-1, a scaffold protein, participates in regulating the activation of Nrf2 signaling pathway by Cx43.

Materials

Penicillin and streptomycin (Cat# V900929) were purchased from Life Technologies TM (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM) (Cat# 41965062) was purchased from Gibco

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Invitrogen Corporation (Gibco, Carlsbad, CA, USA). D-Glucose (Cat# 0188) was purchased from Amresco (Solon, OH, USA). Streptozotocin (STZ) (Cat# S0130) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Nuclear Extract Kit was purchased from Active Motif (Carlsbad, CA, USA). An enhanced chemiluminescence substrate (Cat# WP20005) for the detection of Horseradish peroxidase (HRP) was obtained from Thermo Fisher Scientific, Inc. (Rockford, USA). Total SOD (Cat#S0101) Assay Kit with WST-8 and Lipid Peroxidation MDA (Cat#S0131) Assay Kit were purchased from Beyotime (Haimen, China).

Antibodies against fibronectin (Santa Cruz Biotechnology Cat# sc-8422, RRID:AB_627598), Connexin 43 (Cx43; (Santa Cruz Biotechnology Cat# sc-271837, RRID:AB_10707826), CKIP-1 (Santa Cruz Biotechnology Cat# sc-376355, RRID:AB_10989604), Connexin 43 (Cell Signaling Technology Cat# 3512, RRID:AB_2294590),*HO-1* (Proteintech Cat# 10701-1-AP, RRID:AB_2118685), SOD1 (Proteintech Cat# 10269-1-AP, RRID:AB_2193750), Nrf2 (Proteintech Cat# 16396-1-AP, RRID:AB_2782956),*Keap1* (Proteintech Cat# 10503-2-AP, RRID:AB_2132625), α-Tubulin (Proteintech Cat# 66031-1-Ig, RRID:AB_11042766), Lamin B1 (Abcam Cat# ab133741, RRID:AB_2616597). HRP-conjugated secondary antibodies were acquired from Promega Corporation (Madison, USA).

Methods

Animals

All animal care and experimental procedures complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996), and are in accordance with the China Animal Welfare Legislation, as well as approved by the Ethics Committee on the Care and Use of Laboratory Animals of Sun Yat-sen University, Guangzhou, China. Animal studies were carried out in line with the ARRIVE guidelines and with the recommendations made by the BJP (Kilkenny et al., 2011; McGrath et al., 2015).

CKIP-1$^{-/-}$ mice on a C57BL/6 background, prepared as previously described (Lu et al., 2008), were kindly provided by Professor Lingqiang Zhang from Skate Key Laboratory of Proteomics, National Center for Protein Sciences, Beijing 100850, China.*CKIP-1*$^{+/-}$ mice were backcrossed with C57BL/6 mice to generate *CKIP-1*$^{+/+}$ (WT) and*CKIP-1*$^{-/-}$ (KO) mice. Mice were genotyped by PCR analysis from DNA obtained from tail biopsies according to the report (Lu et al., 2008). *CKIP-1*$^{+/+}$ (WT) and*CKIP-1*$^{-/-}$ (KO) mice (male, 6–8 weeks old, weighing 20 ± 2 g, SPF grade) were used for subsequent experiments.

All animals were housed under specific pathogen-free (SPF) conditions and in a temperature-controlled (20–25 degC) and humidity-controlled (40–70%) barrier system with a 12 h: 12 h light and dark cycle.

Mouse model of diabetic nephropathy

Diabetic mice were induced according to the method reported(Nath et al., 2017). Specifically, after a week of adaptive feeding, the mice were fed a high-fat diet (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal as per) for 2 weeks, and then intraperitoneally injected with freshly prepared STZ (40 mg*kg^{-1}, dissolved in freshly prepared ice-cold 0.1 M citrate buffer, pH 4.5) for five consecutive days after fasted 8 h in continuation with a high-fat diet to induce diabetic mice. The control mice were provided with a normal standard balanced diet, followed by intraperitoneal injection with the same volume of citrate buffer. Two weeks after STZ injection, the fasting blood glucose (FBG) of STZ-induced mice was detected by a One-Touch glucometer (Johnson and Johnson, USA), and mice with FBG levels above 11.1 mM were considered as diabetic mice.

Wild-type (WT) and *CKIP-1*$^{-/-}$ (KO) mice were modeled separately, and the mice was divided into four groups as follows: WT-Ctrl (n=16), KO-Ctrl (n=10), WT-DM (n=16), KO-DM (n=16).

Tail vein injection of recombinant Cx43 adenovirus

Recombinant Cx43 adenovirus (Ad-Cx43; Genbank ID NM_010288, Genechem, Shanghai, China). Sixteen diabetic mice (DM) were randomly divided into two groups, which were given tail-vein injection of negative control adenovirus (DM + Ad-V, n=8) or adenovirus-mediated Cx43 overexpression (DM + Ad-Cx43, n=8).

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Similarly, the control mice (Ctrl) were randomly divided into negative control adenovirus group (Ctrl + Ad-V, n=8) and adenovirus-mediated Cx43 overexpression group (Ctrl + Ad-Cx43, n=8). *CKIP-1*$^{-/-}$ (KO) mice were grouped according to the following: Ctrl + Ad-V (n=10), DM + Ad-V (n=8), DM + Ad-Cx43 (n=8), which were injected with negative control adenovirus or adenovirus-mediated Cx43 overexpression. In compliance with the method tested before (Chen et al., 2019), the mice were administered with Cx43 adenovirus (Ad-Cx43) (2 x 10^8 PFU, 200 μL per mouse) or negative control adenovirus (Ad-V) (2 × 10^8 PFU, 200 μL per mouse) weekly via tail vein injection. The change of FBG and body weight of mice was measured once a week using a One-Touch blood glucose meter after starvation for 12 h and treated with adenovirus for 8 consecutive weeks.

Biochemical analysis

At the termination of the experiment, the FBG levels and body weight of mice were recorded, and the urine was collected from the mice housed in metabolic cages for 24 h. All mice were sacrificed after anesthesia, then blood samples were collected and separated serum and stored at -20°C until used for biochemical analysis. Kidneys samples were quickly excised, weighed, and fixed in 4% paraformaldehyde, or frozen in liquid nitrogen and then stored at -80°C. The measurement of glycated serum protein (GSP), blood urea nitrogen (BUN), serum creatinine (Cr) and 24 h-urinary protein (UP) level were detected by using commercially-available kits (Jiancheng biotech, China). In addition, the level of malondialdehyde (MDA) and the activity of total superoxide dismutase (SOD) in serum and kidney tissues were also measured with the commercially-available kits (Beyotime, China).

After fixed with 4% paraformaldehyde and embedded with paraffin, sections of kidney (4 μm thick) were stained with PAS, HE and Masson. The results were photographed by light microscope and the images were converted into digital images to analyze the pathological changes of the glomerulus. In addition, the immunohistochemical and immunofluorescence experiments of kidney tissue sections were performed by Guangzhou Sevier Biology Company.

Cell culture

Primary glomerular mesangial cells (GMCs) were separated from the kidney cortex fragments of male Sprague Dawley (SD) rats (150-180 g) and cultured using standard protocols as previously described (Chen et al., 2019). Briefly, the cortex fragments were cut into pieces and mechanically passed through specific mesh sizes (175, 147, and finally 74 μm). The tissue (containing the glomeruli) was then digested with 0.1% collagenase (Type IV; U·mL^{-1}) in DMEM for 15–25 min at 37degC. Finally, the digested cells were cultured in DMEM medium containing 20% fetal calf serum (FBS), 0.66 U*mL^{-1}of insulin, 2-mM L-glutamine, 100 U*mL^{-1} of penicillin, and 100 U*mL^{-1} streptomycin at 37degC in a 5% CO_2 incubator. The culture medium was not replaced until the fourth day and was renewed every 2 or 3 days until about 30 days. GMCs ranging from passages 5 to 13 were used for the experiments. The GMCs were cultured in DMEM with 10% FBS at 37degC in a 5% CO_2 incubator. Before treatment, the GMCs were serum-starved for 16 h and then treated with glucose (5.6 mM as normal glucose and 30 mM as high glucose).

Transfections of plasmids and small-interfering RNA

Plasmid: msfGFP-Cx43 (RRID: Addgene_69024) was obtained from Addgene (Watertown, MA, USA). CMV-Cx43-HA (1-382aa) and CMV-Cx43ΔCT-HA (1-234aa) (a plasmid that lacked the Cx43 C-terminus) were purchased from GeneChem (Shanghai, China). GMCs were plated in 35 mm culture plates for 24 h before transfection of plasmids. After transfection with 2 μg indicated plasmid using Lipofectamine ® LTX & Plus Reagent, GMCs were incubated for 72 h to harvest, and western blot assay was performed.

Small-interfering RNA (siRNA) of CKIP-1 was purchased from GenePharma (GenePharma, shanghai, China). The following were the special sequences of CKIP-1-siRNA: sense: CUCCGGAAAUCCAAGAGUATT and antisense: UACUCUUGGAUUUCCGGAGTT. GMCs were cultured in 35 mm plates 24 h prior to transfection, then 5 μL siRNA (50 nM) and 5 μL Lipofectamine ® RNAiMax Reagent (1:1 ratio) were transfected

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into cells according to the protocol. After further treatment, the cells were harvested and subjected to Western blot assay.

Western blot assay

The antibody-based procedures used in this study was complied with the recommendations made by the British Journal of Pharmacology. Western blot assay was consistent with the previously described procedures (Z. Chen et al., 2017) and complied with BJP guidelines and the editorial on reporting of such studies (Steve P. H. Alexander et al., 2018).In summary, kidney tissue homogenates or GMCs were extracted using RIPA lysis buffer with protease inhibitor cocktail, phosphatase inhibitor A and B for 30 min. After centrifuged at 12,000g for 15 min at 4 °C, protein concentration was determined using a BCA Protein Assay Kit (Pierce, USA). Then, an equal amount of protein samples was separated using SDS-PAGE, and then transferred to PVDF membranes (Bio-rad Laboratories, USA). After blocking with 5% non-fat dry milk in 0.1% Tween-20/TBS (TBST) for 1 h at room temperature, the blots were incubated overnight at 4 degC with primary antibodies. After further washing, the membranes were incubated for 1 h at room temperature with the corresponding HRP-conjugated secondary antibodies (Promega Corporation, Madison, WI, USA). Finally, immunoreactive bands were visualized with GE ImageQuant LAS4000mini (GE healthcare, Waukesha, WI, USA ImageQuant, RRID: SCR_014246), and then analyzed using Quantity One Protein Analysis Software (Bio- Rad, Hercules, CA, USA; Version 4.6.2, RRID: SCR_016622).

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

The EMSA assay was carried out to detect the binding activity of Nrf2 and antioxidant reaction element (ARE) following the protocol of the manufacturer. As previously described (W. Y. Gong et al., 2017), nuclear proteins and cytoplasmic proteins were isolated using nuclear extracts prepared by a Nuclear Extract Kit (Active Motif, USA) from different groups of cells. The extracted nuclear proteins (5 μg) were incubated with 50 $ng{\cdot}mL^{-1}$ poly (dI-dC), 0.05% Nonidet P-40, 5 mM $MgCl_2$, and 2.5% glycerol (Light Shift Chemiluminescent EMSA Kit; Pierce, USA) for 10 min. Subsequently, incubation at room temperature for 20 min with 0.2 pmol of biotin-labeled ARE consensus oligonucleotide (Beyotime, China) was performed. The reaction mixture was then subjected to 7% non-denaturing SDS–PAGE, transferred onto a nylon hybridization transfer membrane (Amersham, USA), and DNA cross-linked for 10 min. After blocked in a blocking buffer for 1 h at room temperature, the blots were incubated with horseradish peroxidase-conjugated streptavidin antibodies (1:300) for 15 min. Finally, the peroxidase activity was detected using an enhanced chemiluminescence substrate system. The images were captured and quantified using GE ImageQuant LAS4000mini.

Immunoprecipitation assay

Immunoprecipitation assays were carried out as previously described (W. Gong et al., 2016), GMCs were lysed with immunoprecipitation lysate buffer on ice for 30 min, and then centrifuged at 12,000g for 15 min at 4 degC. Preclear reaction was conducted by incubating whole-cell lysates (400 μg) with 20 μL of protein agarose A/G beads to reduce nonspecific combination, followed by transient centrifugation to collect the supernatants. The supernatants were then incubated overnight with 2 μg of mouse IgG or Cx43, CKIP-1 or Nrf2 antibodies at 4 °C with shaking. About 20 μL of protein agarose A/G beads were added to the supernatants, and the mixture was shaken for 2 h at 4 °C. After centrifuging at 4 °C, 12,000g for 30 s, the supernatant was carefully divided without touching the beads. And then the beads were extensively washed three times with immunoprecipitation buffer 1, 2 and 3, and about 25 μL of SDS-PAGE loading buffer were added to the beads, after which the mixture was boiled for 5 min twice. The immunoprecipitates were resolved by 8% SDS-PAGE followed by Western blot analysis with respective antibodies.

Immunofluorescence staining

The procedure of immunofluorescence staining was consistent with the previous study (W. Y. Gong et al., 2017). GMCs were grown on glass coverslips in 24-well plates. After treated with corresponding stimuli, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at room temperature, dried at room temperature for 10 min, permeabilized with 1% Triton X-100 in PBS for

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10 min, and then blocked with goat serum for 1 h. Then, the cells were incubated overnight with anti-Cx43 (for CKIP-1 subcellular detection), anti-CKIP-1 (for CKIP-1 subcellular detection) and anti-Nrf2 (for Nrf2 translocation assay) antibodies in goat serum and then coverslips were washed with PBS and incubated in the dark with secondary antibody (Alexa Fluor 488 and Alexa Fluor 594, Invitrogen, Carlsbad, CA) at room temperature for 1 h. The nuclei were colabeled with DAPI solution (5 $mg{\cdot}mL^{-1}$ in PBS, Sigma, St. Louis, MO) for 10 min in the dark at room temperature. Finally, the coverslips were washed and mounted on slides with anti-fade mounting medium (Beyotime, Haimen, China). The images were collected using a Zeiss LSM 510 laser confocal fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Detection of intracellular superoxide and H_2O_2 levels

The levels of superoxide and H_2O_2 in GMCs were detected to monitor oxidant formation. The fluorescent probe Dihydroethidium (DHE, Beyotime, Haimen, China) was used to mainly detect intracellular superoxide levels. After various treatments, cells were washed twice with PBS and then added with DHE (10 μM) in DMEM without serum for 30 min at 37 °C. The fluorescence was then measured with a high content screening (HCS, ArrayScan VTI 600 plus, Thermo Fisher, Rockford, USA). Meanwhile, intracellular H_2O_2 level was measured by Hydrogen Peroxide Assay Kit (Beyotime, Haimen, China). Firstly, the sample and schizolysis solution supplied by the kit were mixed at a ratio of 1:1, and then after centrifuged at 1100g for 10 min, the supernatants were collected for further tests. All the operations were carried out on ice. At last, the test tubes containing 50 μL of supernatants and 100 μL of test solutions were placed at room temperature for 20 min and measured instantly with a spectrometer at a wavelength of 560 nm. The concentration of H_2O_2 released was calculated according to standard concentration curve originated from standard solutions upon the identical experiments.

Data and statistical analysis

The data and statistical analysis comply with the recommendations of the BJP on experimental design and analysis in pharmacology (Curtis et al., 2018). All the studies were designed to generate groups of equal size, using randomization and blinded analysis. Statistical analysis was undertaken only for studies where each group size was at least $n = 5$ using GraphPad Prism 5.0 (GraphPad Prism, RRID: SSCR_002798). The values were expressed as mean ± SD.

Sample sizes in each group subjected to statistical analysis were determined based on our previous studies, preliminary results, and the power analysis (Curtis et al., 2015). Additionally, group size is the number of independent values and that statistical analyses used these independent values (i.e., not treating technical replicates as independent values). The values of the control group were normalized to 100% in western blotting analysis to reduce unwanted sources of variation, which was analyzed by non-parametric tests (Kruskal-Wallis test followed by Dunn's multiple comparison). The Y-axis shows the percentage of the treated group to that of the corresponding matched control values. Outliers were excluded according to a predefined and defensible set of exclusion criteria (such as the maximum and minimum) in the animal experiments. Unpaired Student's t-test was used to compare two groups. For multiple comparisons, the data were analyzed by one-way ANOVA with post hoc multiple comparisons. Post hoc tests were further conducted only if the F was significant and there was no significant variance inhomogeneity. $P<0.05$ was considered to be statistically significant, and this P value was not varied later.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Stephen P. H. Alexander, Fabbro, et al., 2017; Stephen P. H. Alexander, Kelly, et al., 2017).

Results

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Effects of Cx43 adenovirus infection on renal fibrosis and renal function parameters in STZ-induced diabetic mice

Firstly, the effect of Cx43 overexpression on renal fibrosis was demonstrated by Cx43 adenovirus infection in STZ-induced diabetic mice. Compared with the control group (Ctrl + Ad-V), the representative HE and PAS staining results of diabetic mice (DM + Ad-V) showed significant glomerular hypertrophy, regional adhesion of the glomerular tuft to the Bowman's capsule, expanded mesangial regions, and obvious increase in the glomerular mesangial matrix index (Supporting Information. S1A), indicating that the diabetic mice had developed renal pathological damage. The injection of adenovirus-mediated Cx43 overexpression (Ad-Cx43) significantly ameliorated the above renal pathological changes in diabetic mice, and had no effect on the control mice (Fig. 1A). Masson staining also revealed that overexpression of Cx43 decreased the generation of collagenous fibers in the glomeruli of diabetic mice and suppressed the pathological process of renal fibrosis.

By the immunohistochemical staining (Fig. 1B) and Western blotting results of Cx43 and FN in kidney tissues (Fig. 1C), the expression of Cx43 in diabetic mice (DM + Ad-V) was significantly lower than that of control mice (Ctrl + Ad-V), whereas the expression of extracellular matrix protein FN was prominently increased. Treatment of adenovirus-mediated Cx43 overexpression could notably improve the protein levels of Cx43 while statistically significantly inhibiting the deposition of FN in the glomeruli of diabetic mice (Supporting Information. S1B).

In addition, the effect of Cx43 overexpression on renal function of diabetic mice was investigated by detecting the fasting blood glucose (FBG, Fig. 1D), glycated serum protein (GSP, Fig. 1E), urea nitrogen (BUN, Fig. 1F), serum creatinine (Cr, Fig. 1G), 24-hour urinary protein (24 h UP, Fig. 1H). Treated with adenovirus-mediated Cx43 overexpression (DM + Ad-Cx43) could remarkably reverse the abnormal up-regulation of FBG, GSP, BUN, Cr, 24 h UP in diabetic mice (DM + Ad-V), which suggested that the overexpression of Cx43 could improve the renal physiological function of diabetic mice.

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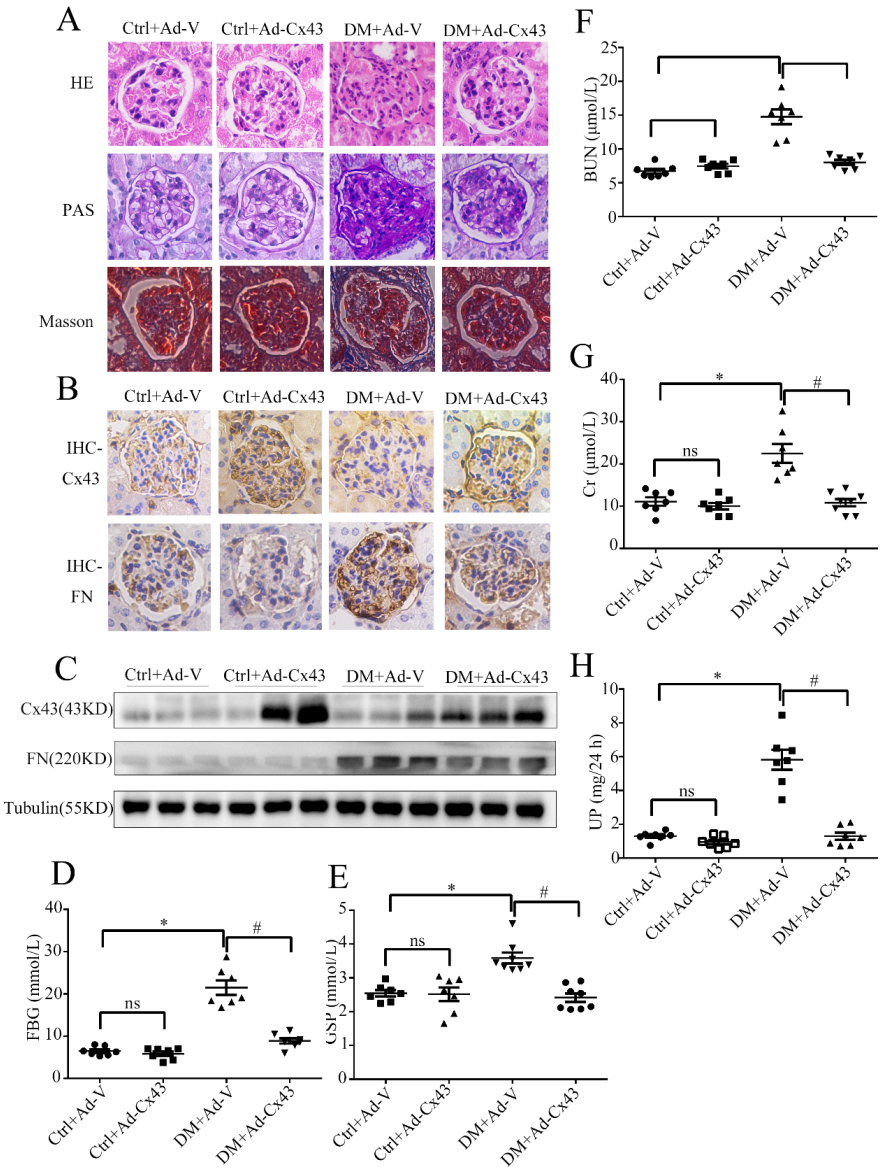

Fig. 1.Effects of Cx43 adenovirus infection on renal fibrosis and renal function parameters in STZ-induced diabetic mice.

(A) Glomerular histopathology analysis was performed by PAS, HE and Masson staining (400 × magnification). (B) The expression of Cx43 and FN in the glomeruli were shown by immunohistochemical staining (400 × magnification). (C) The levels of Cx43 and FN in kidney tissues of diabetic mice were detected using the Western blot assay. (D) The FBG of diabetic mice were analyzed. (E) The Serum BUN of diabetic mice were detected to assess renal injury. (F) The Serum Cr of diabetic mice were detected to assess renal injury. (G) The 24 h UP were detected to assess renal injury. The data are presented as the means ± SD; n = 7 (one outlier was excluded). $^{*}P < 0.05$, significantly different from Ctrl + Ad-V; $^{\#}P < 0.05$, significantly different from DM + Ad-V. Ad-Cx43: Cx43 adenovirus; Ad-V: vector adenovirus. Ctrl: control; DM: diabetes mellitus; FBG: fasting blood glucose; BUN: blood urea nitrogen; Cr: serum creatinine; UP 24 h: urine protein for 24 h.

Treatment with Cx43 adenovirus enhanced the activation of Nrf2 signaling pathway

We further demonstrated that the effect of adenovirus-mediated Cx43 overexpression on diabetic renal fibrosis was associated with its activation of Nrf2 anti-oxidative stress signaling pathway in diabetic mice. Treatment with Cx43 adenovirus could promote the nuclear translocation of Nrf2 and activate the Nrf2 signaling pathway in the glomeruli of diabetic mice (Fig. 2A). By detecting the protein levels of Nrf2 as well as its two target genes HO-1 and SOD1 in the kidney tissues, we found that overexpression of Cx43 could evidently reverse the reduction of Nrf2, HO-1 and SOD1 in the kidney tissues of diabetic mice (Fig. 2B, Supporting Information. S1C).

Moreover, injection of adenovirus-mediated Cx43 overexpression could not only reverse the increased MDA levels (Fig. 2C and D), but also enhance the total SOD activity in serum and kidney tissues of diabetic mice (Fig. 2E and F), which inhibited the renal oxidative damage of diabetic mice.

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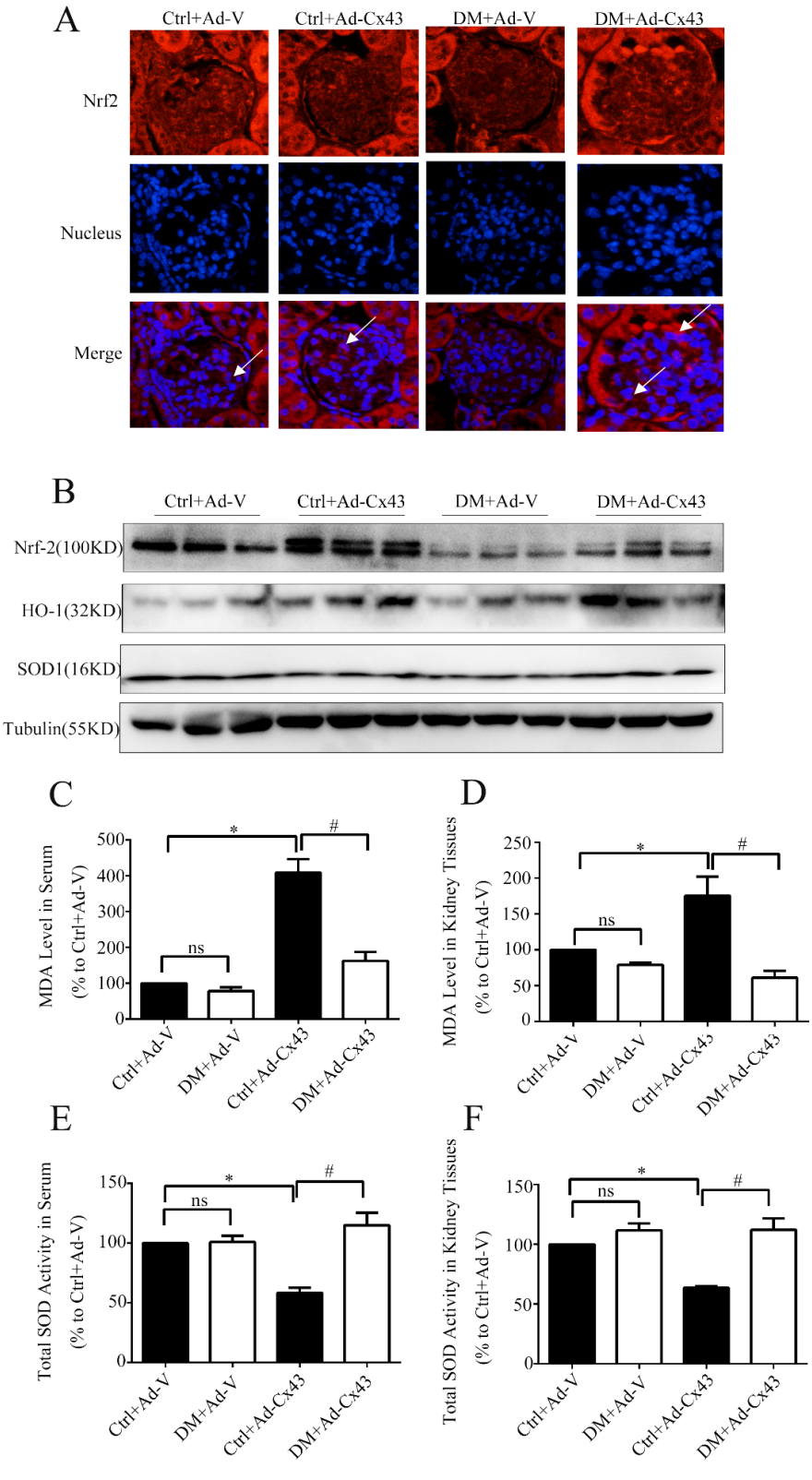

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Fig.2.

Treatment with Cx43 adenovirus enhanced the activation of Nrf2 anti-oxidative stress signaling pathway

(A) Immunofluorescence staining revealed that the nuclear translocation of Nrf2 in the glomeruli of diabetic mice by Cx43 adenovirus treatment (400 × magnification). (B)Western blot results showed that Cx43 adenovirus reversed the down-regulated expression of Nrf2, HO-1 and SOD1 in the kidney tissues of diabetic mice. (C and D) Cx43 adenovirus decreased the MDA levels in the serum and kidney tissues of mice. (E and F) Cx43 adenovirus increased the total SOD activity in the serum and kidney tissues of mice. The data are presented as the means ± SD; n = 6.*P < 0.05, significantly different from Ctrl + Ad-V; $^{\#}$P < 0.05, significantly different from DM + Ad-V. Ad-Cx43: Cx43 adenovirus; Ad-V: vector adenovirus. Ctrl: control; DM: diabetes mellitus.

CKIP-1 deficiency aggravated diabetic renal fibrosis and oxidative damage in STZ-induced diabetic mice

CKIP-1$^{-/-}$ mice were intraperitoneally injected STZ to induce diabetic mice models. The results of pathological staining including HE, PAS and Masson staining (Fig. 3A) showed that compared with diabetic mice (DM), *CKIP-1*$^{-/-}$ diabetic mice (KO-DM) had developed more severe glomerular pathological changes, higher glomerular mesangial matrix index (Supporting Information. S2A) and further deteriorated renal fibrosis. The deficient expression of CKIP-1 was confirmed in the kidney tissues of *CKIP-1*$^{-/-}$ diabetic mice (KO-DM) by the immunohistochemical staining and Western blotting results whereas the protein levels of FN were markedly increased (Fig. 3B and C). We also detected the level of renal oxidative stress in *CKIP-1*$^{-/-}$ diabetic mice and found that the expression of Nrf2, HO-1 and SOD1 in the kidney tissues were significantly suppressed (Fig. 3C, Supporting Information. S2B), while the MDA levels in the serum and kidney tissues further increased (Fig. 3D and E) with the further inhibited total SOD activity (Fig. 3F and G), indicating CKIP-1 deficiency aggravated renal oxidative damage in diabetic mice.

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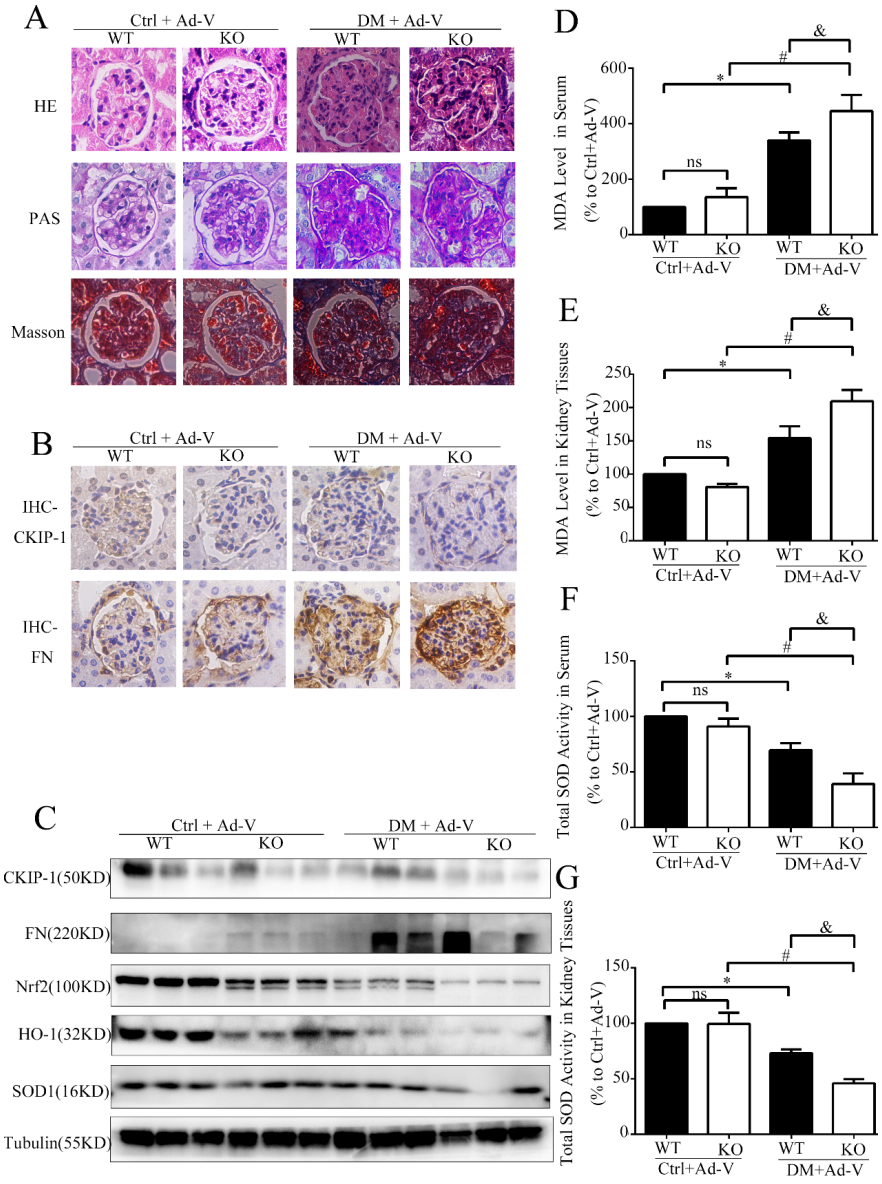

Fig.3. CKIP-1 deficiency aggravated diabetic renal fibrosis and oxidative damage in STZ-induced diabetic mice

(A) Glomerular histopathology analysis was performed by PAS, HE and Masson staining (400 × magnification). (B) The expression of CKIP-1 and FN in the glomeruli were shown by immunohistochemical staining (400 × magnification). (C) The deficiency of CKIP-1 further promoted the accumulation of FN but reduced the expression of Nrf2, HO-1 and SOD1 in kidney tissues of diabetic mice by the Western blot assay. (D and E) The MDA levels in the serum and kidney tissues were higher in *CKIP-1*$^{-/-}$ diabetic mice than that in diabetic mice. (F and G) The total SOD activity in the serum and kidney tissues was lower in *CKIP-1*$^{-/-}$ diabetic mice than that in diabetic mice. The data are presented as the means +- SD; n = 6.*P < 0.05, significantly different from WT-Ctrl+Ad-V; $^{\#}$P < 0.05, significantly different from KO-Ctrl+Ad-V; $^{\&}$P < 0.05, significantly different from WT-DM+Ad-V. WT: wide type; KO: knock out; Ctrl: control; DM: diabetes mellitus.

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Injection of Cx43 adenovirus could not improve diabetic renal fibrosisin STZ-induced *CKIP-1*$^{-/-}$ diabetic mice

Subsequently, we retested the effect of adenovirus-mediated Cx43 overexpression on diabetic renal fibrosis in STZ-induced *CKIP-1*$^{-/-}$diabetic mice by tail vein injection. However, it was found that the overexpression of Cx43 did not effectively ameliorate the renal pathological damage, nor could it down-regulate the glomerular mesangial matrix index (Supporting Information. S3A) or inhibit the development of renal fibrosis in *CKIP-1*$^{-/-}$ diabetic mice (Fig. 4A). The treatment of adenovirus-mediated Cx43 overexpression in *CKIP-1*$^{-/-}$ diabetic mice significantly increased the expression of Cx43, but failed to down-regulate the protein levels of FN in kidney tissues (Fig. 4B and C, Supporting information.S3B). Similarly, through the detection of the levels of FBG, GSP, BUN, Cr, 24 h UP (Fig. 4D, E, F, G and H), we also demonstrated that the injection of Cx43 adenovirus could not effectively reverse the abnormalities of above kidney physiological indicators, indicating the loss of renal protective effect of Cx43 adenovirus in STZ-induced *CKIP-1*$^{-/-}$ diabetic mice.

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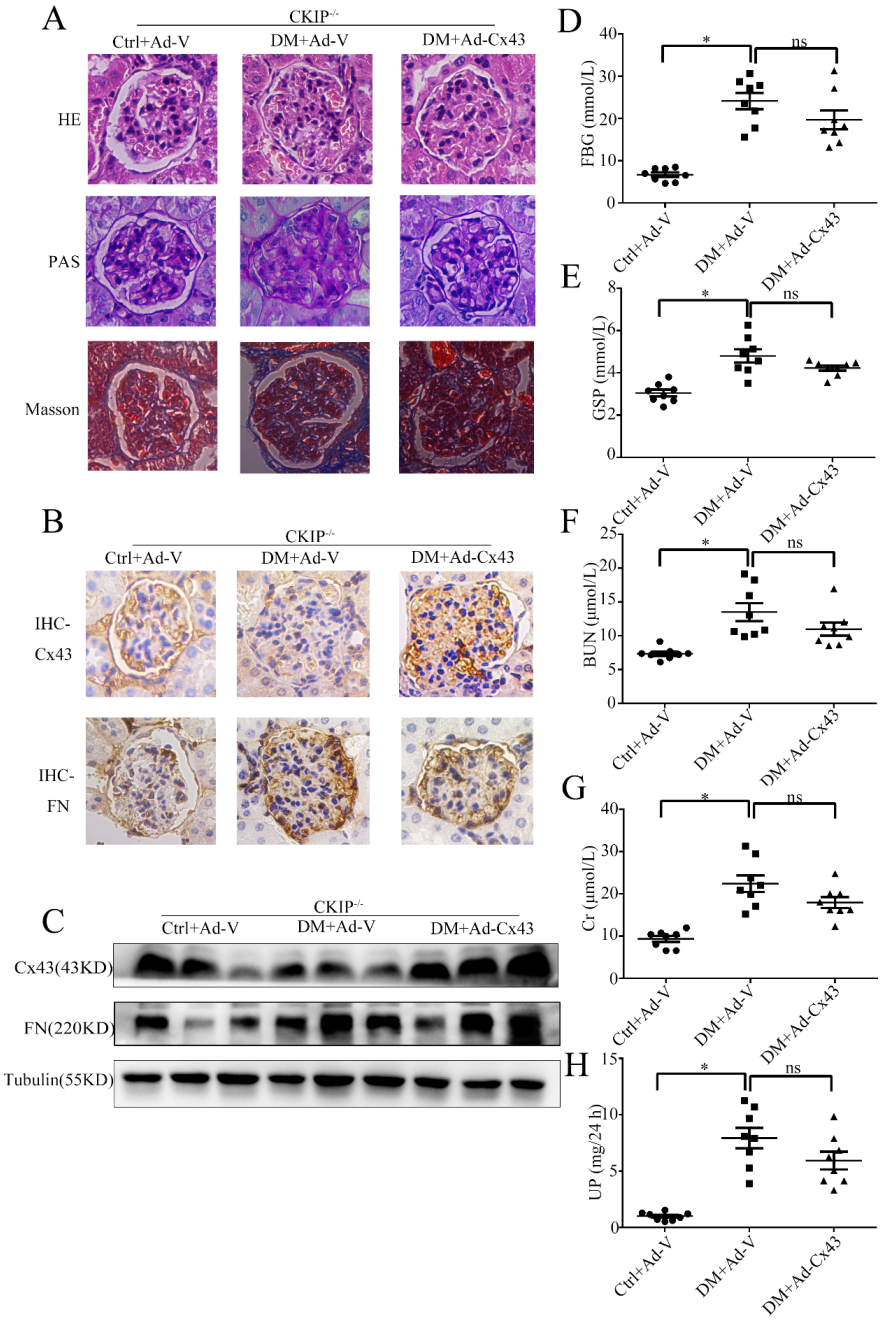

Fig.4.Injection of Cx43 adenovirus could not improve diabetic renal fibrosis in STZ-induced *CKIP-1*$^{-/-}$ diabetic mice

(A) Glomerular histopathology analysis was performed by PAS, HE and Masson staining (400 × magnification). (B) The expression of Cx43 and FN in the glomeruli were shown by immunohistochemical staining (400 × magnification). (C) The levels of Cx43 and FN in kidney tissues of *CKIP-1*$^{-/-}$ diabetic mice were detected using the Western blot assay. (D) The FBG of *CKIP-1*$^{-/-}$ diabetic mice were analyzed. (E) The Serum BUN was detected to assess renal injury. (F) The Serum Cr were detected to assess renal injury. (G) The 24 h UP were detected to assess renal injury. The data are presented as the means +- SD; n = 8 (two outliers were

excluded). $^{*}P < 0.05$, significantly different from Ctrl + Ad-V. Ad-Cx43: Cx43 adenovirus; Ad-V: vector adenovirus. Ctrl: control; DM: diabetes mellitus; FBG: fasting blood glucose; BUN: blood urea nitrogen; Cr: serum creatinine; UP 24 h: urine protein for 24 h.

Injection of Cx43 adenovirus could not induce the activation of Nrf2 signaling pathwayin *CKIP-1*$^{-/-}$ diabetic mice

Furthermore, the disability of adenovirus-mediated Cx43 overexpression to activate the Nrf2 anti-oxidative stress signaling pathway in *CKIP-1*$^{-/-}$ diabetic mice was also discussed. Compared with *CKIP-1*$^{-/-}$ diabetic mice (DM+ Ad-V), treatment with Cx43 adenovirus (DM+ Ad-Cx43) did not promote the accumulation of Nrf2 in the glomerular nucleus (Fig. 5A), and could not reverse the significantly down-regulated expression of Nrf2, HO-1 and SOD-1 in kidney tissues (Fig. 5B, Supporting Information. S3C). The MDA in the serum and kidney tissues of *CKIP-1*$^{-/-}$ diabetic mice injected with Cx43 adenovirus (DM+ Ad-Cx43) still maintained high levels (Fig. 5C and D) whereas the total SOD activity was still inhibited (Fig. 5E and F), indicating the overexpression of Cx43 could not relieve the oxidative damage in diabetic mice with CKIP-1 deficiency.

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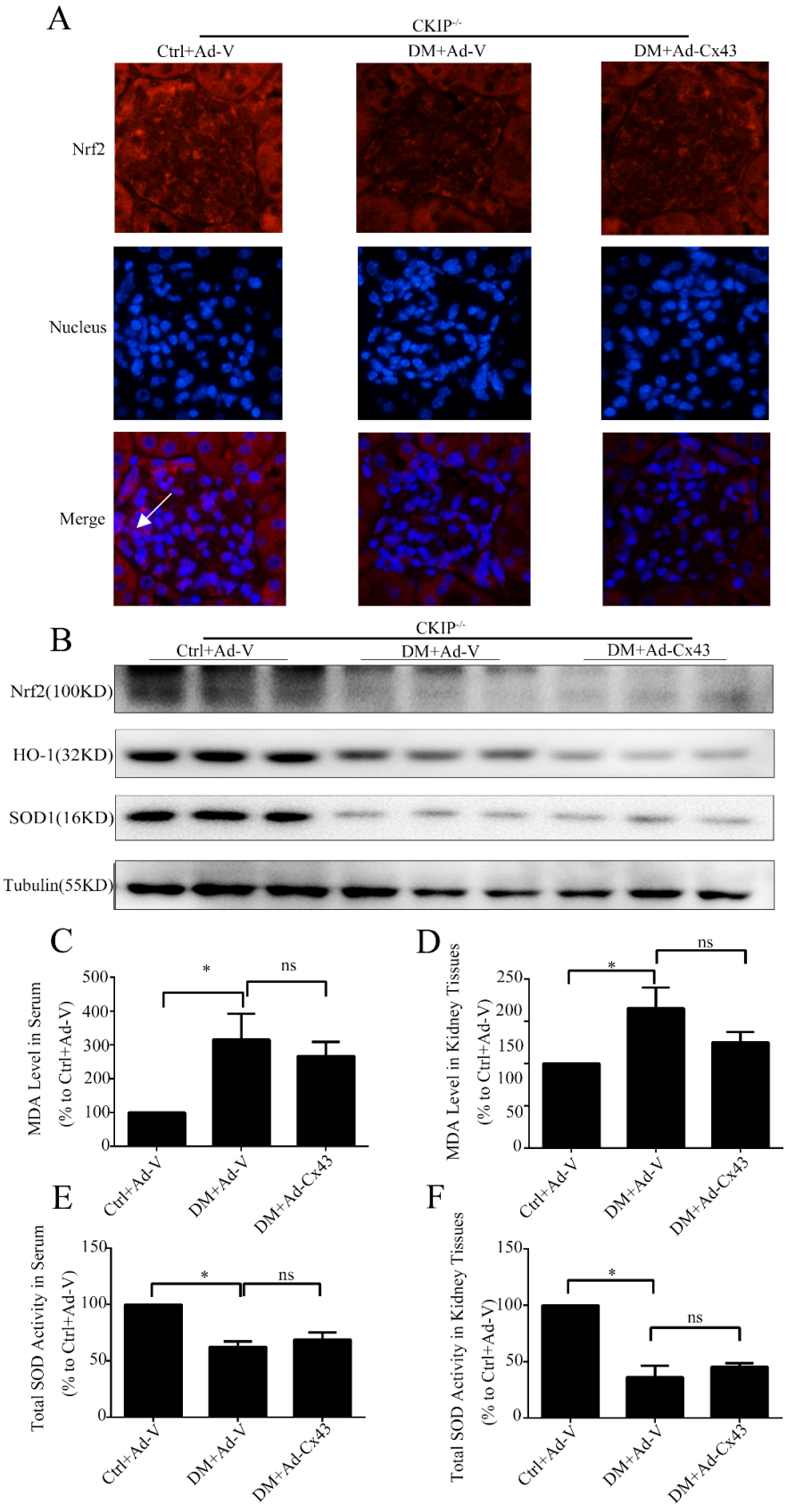

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Fig.5. Injection of Cx43 adenovirus could not induce the activation of Nrf2signaling pathwayin *CKIP-1*$^{-/-}$ diabetic mice

(A) The nuclear translocation of Nrf2 in the glomeruli of *CKIP-1*$^{-/-}$ diabetic mice injected with Cx43 adenovirus was detected by Immunofluorescence staining (400 × magnification). (B)Western blot results showed that Cx43 adenovirus could not improve the expression of Nrf2, HO-1 and SOD1 in the kidney tissues of *CKIP-1*$^{-/-}$ diabetic mice. (C and D) The MDA levels in the serum and kidney tissues of *CKIP-1*$^{-/-}$mice were detected. (E and F) The total SOD activity in the serum and kidney tissues of C *CKIP-1*$^{-/-}$ mice were detected. The data are presented as the means +- SD; n = 6. $^{*}P < 0.05$, significantly different from Ctrl + Ad-V. Ad-Cx43: Cx43 adenovirus; Ad-V: vector adenovirus. Ctrl: control; DM: diabetes mellitus.

The interference of CKIP-1 blocked the activation ofNrf2 signaling pathway by Cx43 in the GMCs cultured in high glucose

To further investigate whether CKIP-1 plays an important role in mediating the activation of Nrf2 signaling pathway by Cx43, both Cx43 overexpressed plasmid and CKIP-1 small interfering RNA (Si-CKIP-1) were simultaneously transfected into GMCs incubated with high glucose. Western blot results (Fig. 6A) confirmed that the effect of Cx43 which reversed the increased expression of FN and Keap1 and the down-regulation of Nrf2, HO-1 and SOD-1 induced by high glucose was counteracted by the transfection of Si-CKIP-1 in GMCs (Supporting Information. S4A).

After interfering with the expression of CKIP-1 in GMCs, the activation of Nrf2 signaling pathway by Cx43 was also inhibited under high glucose conditions. By detecting the expression of Nrf2 in the nucleus and cytoplasm respectively, the distribution of Nrf2 in the nucleus was increased and the expression of Nrf2 in the cytoplasm was reduced by the overexpression of Cx43 (Fig. 6B). EMSA results (Fig. 6C) showed that the overexpression of Cx43 enhanced the binding activity of Nrf2 to ARE and the results of immunofluorescence (Fig. 6D) also found that the nuclear accumulation of Nrf2 was induced by the overexpression of Cx43. However, with the transfection of Si-CKIP-1, the above effects of Cx43 on activating the Nrf2 anti-oxidative stress signaling pathway were all inhibited. Furthermore, results also demonstrated that Cx43 could no longer inhibit the intracellular superoxide and H_2O_2levels (Fig. 6E and F, Supporting Information. S6D) after interfering with the expression of CKIP-1 in GMCs boosted by HG.

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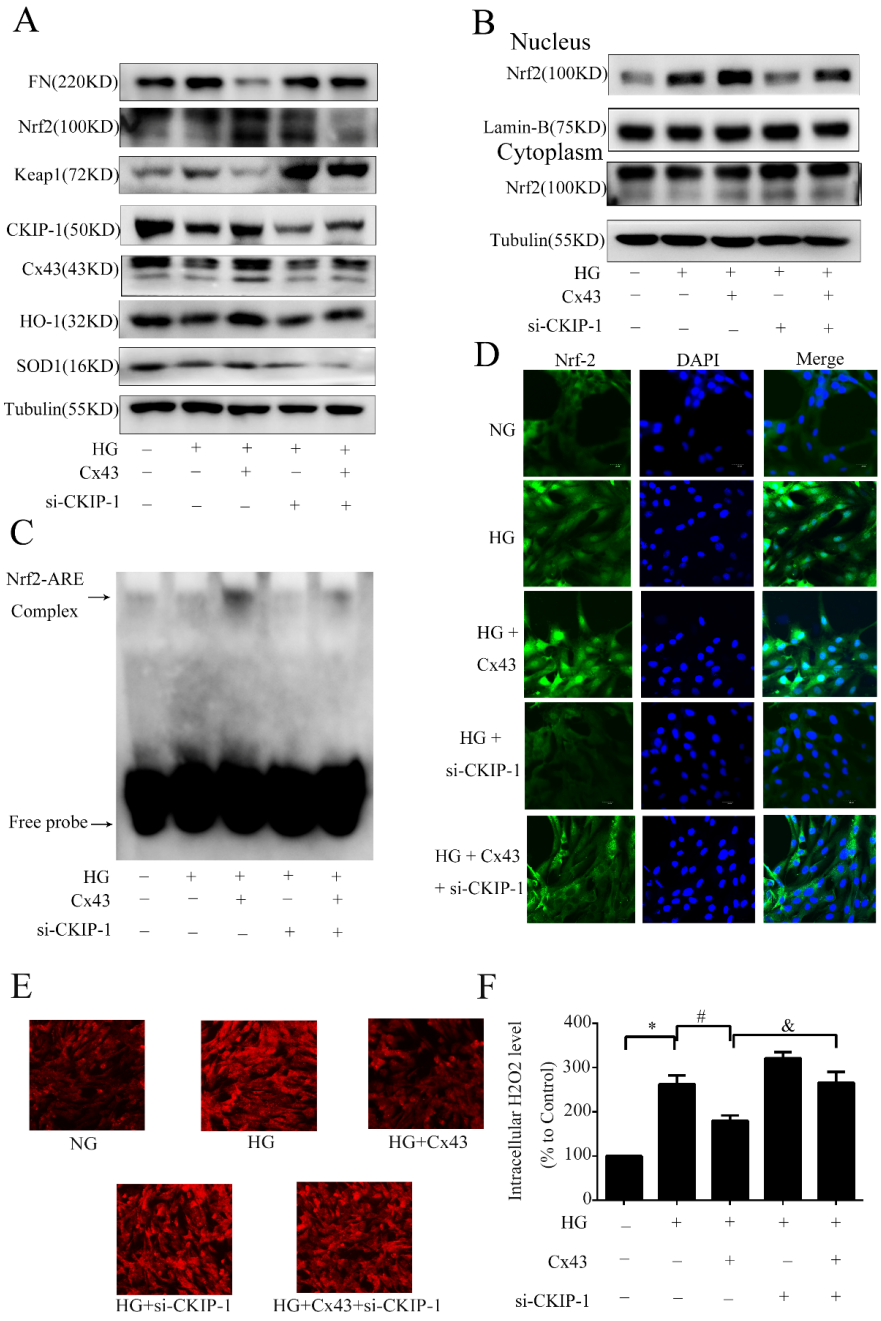

Fig.6.The interference of CKIP-1 blocked the activation of Nrf2 signaling pathway by Cx43 in the GMCs cultured in high glucose

(A)The protein levels of FN, Nrf2, Keap-1, CKIP-1, Cx43, HO-1 and SOD1 were detected by the Western blot assay. (B) The Western blot results shown the expression of Nrf2 in the nucleus and cytoplasm. (C) EMSA was performed to determine the DNA binding activity of Nrf2. (D) The subcellular distribution of Nrf2 in GMCs was measured by immunofluorescent staining. (E) The intracellular superoxide levels were detected using the DHE probe. (F) The H_2O_2 levels in GMCs were detected. The data are presented as the means ± SD; n=5. $^{*}P < 0.05$ significantly different from NG; $^{\#}P < 0.05$ significantly different from HG; $^{\$}P$

< 0.05 significantly different from HG+Cx43.

Cx43 interacted with CKIP-1 via the CT domain of Cx43

Next, we discussed whether Cx43 interacted with CKIP-1 in GMCs. Immunofluorescence results (Fig.7A) showed that GFP-CX43 and RFP-CKIP-1 co-located in the cell membrane and cytoplasm of GMCs. As shown by the immunoprecipitation assay, Cx43 interacted with CKIP-1 in GMCs under physiological conditions while the interaction was weakened by treatment with high glucose (Fig. 7B). Immunoprecipitation results of Cx43 also suggested that transfection of Cx43 overexpressed plasmids (wild type) increased the interaction of Cx43 with CKIP-1 whereas transfection of Cx43 plasmids (CT domain deleted) had no effects (Fig.7D), indicating that Cx43 interacted with CKIP-1 via the CT domain of Cx43.

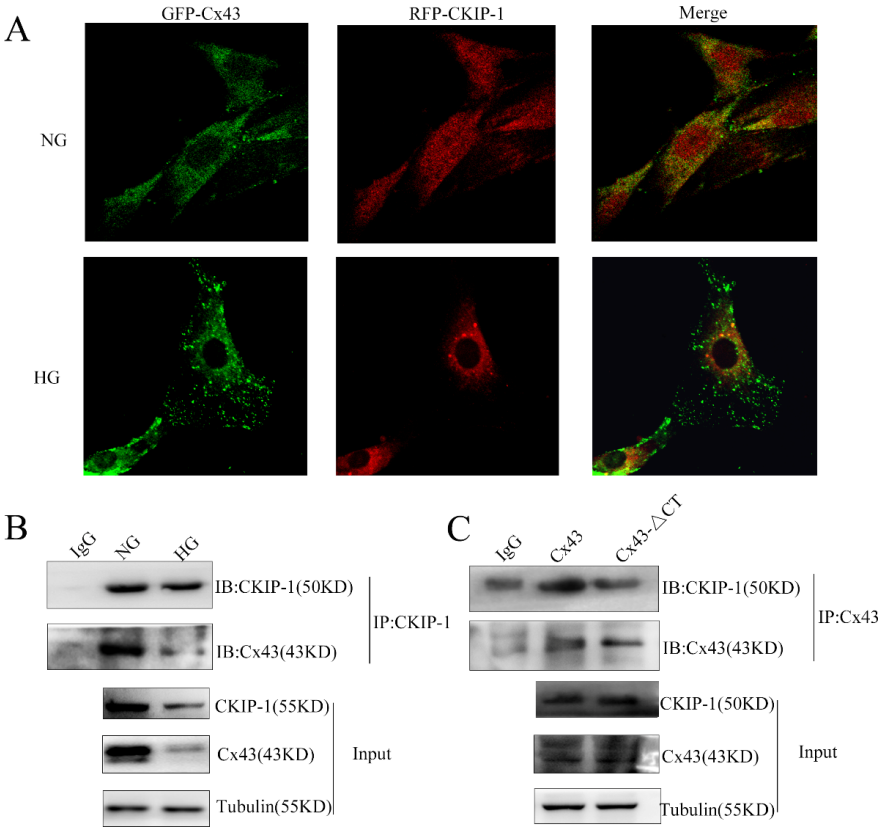

Fig.7. Cx43 interacted with CKIP-1 via the CT domain of Cx43

(A) Cx43 co-localized with CKIP-1in GMCs under normal conditions, as revealed by immunofluorescence assay, but the co-localization of Cx43 with CKIP-1 was reduced by high glucose. Green fluorescence indicates the localization of Cx43. Red fluorescence indicates CKIP-1. (B) Immunoprecipitation assay of CKIP-1 confirmed the interaction between Cx43 and CKIP-1 in GMCs while treatment with high glucose reduced the interaction. (C) Immunoprecipitation results of Cx43 suggested that Cx43 interacted with CKIP-1 via the CT domain of Cx43. Cx43: Cx43 overexpressed plasmids (wild-type); Cx43-CT: Cx43 overexpressed plasmids (CT domain deleted).

Cx43 regulated the expression of CKIP-1 and the interaction of CKIP-1 with Nrf2 through Cx43 CT

Detailed study has revealed the importance of the Cx43 CT regard to both regulation and function of Cx43 (Leithe et al., 2018). Whether Cx43 CT was associated with the regulation of CKIP-1 by Cx43 deserved

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our attention. We found that the expressions of Cx43 and CKIP-1 were significantly decreased in GMCs cultured with high glucose, which could be reversed after transfection with Cx43 overexpressed plasmids (wild-type), suggesting that Cx43 could regulate the expression of CKIP-1 under high glucose conditions (Fig. 8A). However, transfected with Cx43 overexpressed plasmids (CT domain deleted) did not usefully up-regulate the expression of CKIP-1 in GMCs incubated with high glucose (Fig. 8B).

Our previous study have indicated the interaction of CKIP-1with Nrf2 in normal cultured GMCs (W. Gong et al., 2016). In this study, we also confirmed that CKIP-1 interacted with Nrf2 in GMCs under physiological conditions, while the interaction of CKIP-1with Nrf2 was visibly inhibited by the stimulation of high glucose, and the binding of Nrf2 to its inhibitory protein Keap1 enhanced (Fig. 8C).Transfection with Cx43 overexpressed plasmids (wild-type) could promote the interaction of CKIP-1 with Nrf2 but inhibit the binding of Nrf2 to Keap1, as the result of which promoted the nuclear accumulation of Nrf2 (Fig.8D). In contrast, transfected with Cx43 overexpressed plasmids (CT domain deleted) could not play the above role. Based on the above results, Cx43 might regulate the expression of CKIP-1 and the interaction of CKIP-1 with Nrf2 through Cx43 CT.

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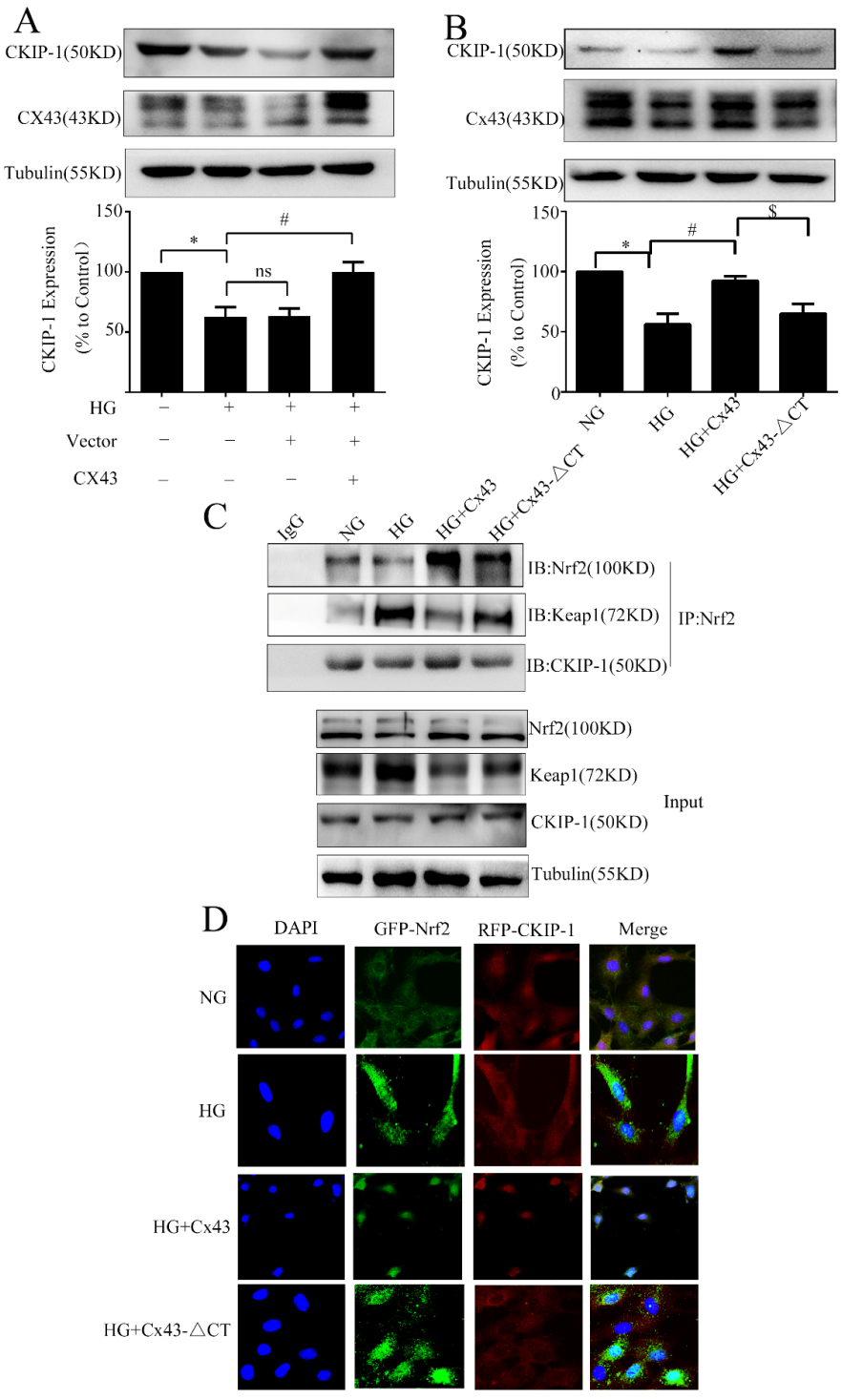

Fig.8. Cx43 regulated the expression of CKIP-1 and the interaction of CKIP-1 with Nrf2 through Cx43 CT

(A)Cx43 regulated the expression of CKIP-1 under high glucose conditions. The data are presented as the means ± SD; n=5. $^{*}P < 0.05$ significantly different from NG; $^{\#}P < 0.05$ significantly different from HG.

(B) Cx43 regulated the expression of CKIP-1 in GMCs incubated with high glucose via the CT domain of Cx43. The data are presented as the means ± SD; n=5. $^{*}P < 0.05$ significantly different from NG; $^{\#}P < 0.05$ significantly different from HG; $^{\$}P < 0.05$ significantly different from HG+Cx43. (C) Immunoprecipitation assay of Nrf2 was used to detect the interaction between Nrf2 and CKIP-1 and the binding of Nrf2 to Keap1 in GMCs. (D) Immunofluorescence results revealed the distribution of Nrf2 and CKIP-1 in GMCs. Green fluorescence indicated the localization of Nrf2. Red fluorescence indicated CKIP-1. Blue fluorescence indicated nuclei. Cx43: Cx43 overexpressed plasmids (wild-type); Cx43-CT: Cx43 overexpressed plasmids (CT domain deleted).

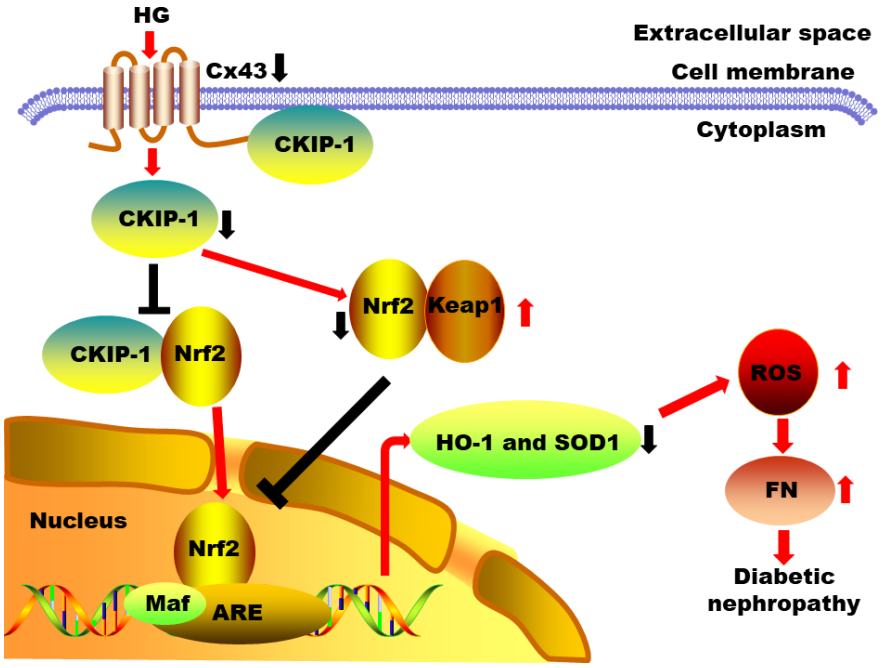

Fig.9. CKIP-1 participates in the activation of Nrf2 signaling pathway by Cx43 and the regulation of diabetic renal fibrosis

Under normal conditions, CKIP-1 was combined with Cx43 via Cx43 CT domain on the membrane of GMCs, which induced the interaction between CKIP-1 and Nrf2 and the nuclear accumulation of Nrf2, leading the activation of Nrf2 anti-oxidative stress signaling pathway. When the cells were stimulated by high glucose, the expression of Cx43 was inhibited, following with the down-regulation of CKIP-1 protein level. Moreover, the interaction between CKIP-1 and Nrf2 was weaken whereas the binding of Nrf2 to its inhibitory protein Keap1 was enhanced, as a result of which the activity of Nrf2 signaling pathway was inhibited with the overproduction of intracellular superoxide. Increased oxidative stress further promoted the expression of FN eventually exacerbating diabetic renal fibrosis.

Discussion

As an important scaffold adaptor, CKIP-1 shuttles between the plasma membrane and the nucleus and functions at different subcellular regions (Fu et al., 2019). A large number of investigations have revealed that the subcellular localization of CKIP-1 highly relies on the cell types and different physiological processes (Olsten et al., 2004; Xi et al., 2010). CKIP-1 binding to various proteins via its different regions could regulate its own subcellular localization status and further control the activity or stability of those interacting proteins, participating in the regulation of signal transduction between the cytoplasm and the nucleus (Lingqiang Zhang et al., 2006; L. Q. Zhang et al., 2005). It was suggested that CKIP-1 could recruit protein phosphatase 2A (PP2A) to regulate the dephosphorylation of histone deacetylase 4 (HDAC4), promote the

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nuclear accumulation of HDAC4 and inhibit the transcription of target genes related to cardiac hypertrophy (Ling et al., 2012). To explore whether CKIP-1 was involved in the activation of Nrf2 anti-oxidative stress signaling pathway by Cx43 and the regulation of pathological process of diabetic renal fibrosis, we detected the mediated effects of CKIP-1 between Cx43 and Nrf2 signaling pathway through *in vivo/in vitro* experiments.

By comparing the effect of adenovirus-mediated Cx43 overexpression on renal fibrosis in wild-type or *CKIP-1*$^{-/-}$diabetic mice, we confirmed that the overexpression of Cx43 significantly improved the renal pathological damage and renal dysfunction by activating the Nrf2 signaling pathway and delayed the pathological progression of renal fibrosis in the glomeruli of diabetic mice; whereas in *CKIP-1*$^{-/-}$ diabetic mice, the effects of adenovirus-mediated Cx43 overexpression on the renal protection and the activation of Nrf2 signaling pathway were all resisted. Similarly, we found that with the transfection of Si-CKIP-1 in GMCs cultured with high glucose, the effect of Cx43 overexpression on the activation of Nrf2 signaling pathway was inhibited. After interfering with the expression of CKIP-1, Cx43 could not promote the nuclear accumulation of Nrf2 and up-regulate the expression of downstream target proteins including HO-1 and SOD1, nor could it inhibit the levels of ROS and the accumulation of fibrosis marker protein FN. Based on the above results, we preliminarily speculated that CKIP-1 might be involved in the activation of Nrf2 anti-oxidative stress signaling pathway by Cx43 and the regulation of diabetic renal fibrosis.

By the immunofluorescence assay, we found that Cx43 and CKIP-1 co-localized in the cell membrane and cytoplasm of GMCs, which was also consistent with the reported subcellular localization of Cx43(Xie et al., 2013)and CKIP-1(W. Gong et al., 2016) in GMCs. Results of immunoprecipitation assay suggested the interaction between Cx43 and CKIP-1 in normal cultured GMCs, and the interaction could be reduced with high glucose stimulation, which provided a subcellular foundation for the regulation between Cx43 and CKIP-1.

Different studies have elucidated the various mechanisms of the activation of Nrf2 signaling pathway upon a series of targets, including directly destroying Nrf2-Keap1 complex, suppressing the degradation of Nrf2 and regulating the cytoplasm-to-nuclear shuttling of Nrf2(Cui et al., 2017). It is well known that when Nrf2 and Keap1 are combined into a complex in the cytoplasm, the nuclear translocation of Nrf2 are inhibited and the degradation of Nrf2 are promoted by ubiquitination, thereby resisting the activation of the Nrf2 signaling pathway (Suzuki et al., 2015; Yamamoto et al., 2018). Directly destroying the combination of Nrf2-Keap1 complex has been proved to be effective as a novel Nrf2 activation strategy (L. Zhou et al., 2015). Our group have previously confirmed that CKIP-1 interacted with Nrf2 in GMCs, and CKIP-1 could affect the polyubiquitination of Nrf2 and Keap1 by regulating Smurf1 to activate the Nrf2 signaling pathway (W. Gong et al., 2018).On this basis, we mainly explored the molecular mechanism by which Cx43 mediated the activation of the Nrf2 pathway by regulating CKIP-1 in the present study.

Cx43 mainly interacts with numerous kinases or scaffold proteins via its CT domain, thereby regulating the biological functions of its binding proteins (Leithe et al., 2018). For example, Cx43 could interact with zona occludens protein-1(ZO-1) though the binding of Cx43 CT to the PDZ-domain of ZO-1 and then link ZO-1 to the cytoskeleton (Barker et al., 2002; Giepmans et al., 1998). In order to investigate whether the regulation of Cx43 on CKIP-1 was associated with Cx43 CT domain, Cx43 overexpressed plasmids (wild type) or Cx43 plasmids (CT domain deleted) were respectively transfected into GMCs and immunoprecipitation results of Cx43 suggested that Cx43 interacted with CKIP-1 via the CT domain of Cx43. Moreover, we also found that transfection of Cx43 overexpressed plasmids (wild type) promoted the expression of CKIP-1 under high glucose conditions, and enhanced the interaction between CKIP-1 and Nrf2 but weakened the binding of Nrf2 to its inhibitor protein Keap1, thereby promoting the nuclear accumulation of Nrf2 and activating the Nrf2 signaling pathway. On the contrary, the above regulatory effect of Cx43 on CKIP-1 was inhibited with the transfection of Cx43 overexpressed plasmids (CT domain deleted), suggesting that Cx43 might regulate the expression of CKIP-1 and the interaction of CKIP-1 with Nrf2 through Cx43 CT. As for the molecular mechanism of how Cx43 regulates the expression of CKIP-1, it might be related to many kinases bound to the CT domain of Cx43, yet to be further explored and clarified.

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Conclusions

Collectively, CKIP-1 serves as an important scaffold adaptor in the activation of Nrf2 anti-oxidative stress signaling pathway by Cx43 and the regulation of the pathological process of diabetic renal fibrosis. The molecular mechanism might be related to Cx43 regulating the expression of CKIP-1 and the interaction of CKIP-1 with Nrf2 through Cx43 CT (Fig.9). Our study provides further experimental basis for targeting the Cx43-CKIP-1-Nrf2 axis to resist diabetic renal fibrosis.

Acknowledgments

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