ATF4 promotes renal tubulointerstitial fibrosis by suppressing autophagy in diabetic nephropathy

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Research

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Abstract

Background

Diabetic nephropathy (DN) is the dominant cause of end-stage renal disease which is characterized by extracellular matrix accumulation. The purpose of this study was to investigate the role of activating transcription factor 4 (ATF4) in regulating renal fibrosis and autophagy in DN.

Methods

Streptozotocin (STZ) was administered to heterozygous ATF4 knockout (KO) and wild-type (WT) mice via an intraperitoneal injection to induce DN. NRK-52E cells were cultured in high glucose to mimic diabetic pathological. qRT-PCR, western blot, immunofluorescence, histology and electron microscopic analysis were performed. The autophagy flow was observed by tandem mRFP-GFP-LC3 fluorescence microscopy.

Results

DN mice experienced severe renal injury and fibrosis and showed increased expression of ATF4 and inhibition of autophagy in kidney tissues. STZ-induced ATF4 KO mice showed significant improvement in urinary albumin, serum creatinine and blood urea nitrogen and the pathological changes of renal tubulointerstitial fibrosis compared with STZ-induced WT mice. Furthermore, western blot assays and immunofluorescence staining revealed that inhibition of ATF4 could restore autophagy in DN mice. Overexpression of ATF4 in NRK-52E cells cultured in high glucose condition suppressed autophagy and upregulated Col-IV expression, while inhibition of ATF4 could increase the number of the autophagosomes, improve autophagic flux and decrease Col-IV level.

Conclusion

Our study provided the first evidence of a crucial role for ATF4 in inhibiting autophagy against diabetic kidney damage. Suppression of ATF4 may be an effective therapy in restraining renal tubulointerstitial fibrosis in DN.

Background

Diabetic nephropathy (DN) has become the main cause of end-stage renal disease worldwide [1]. Tubule interstitial fibrosis (TIF), characterized by tubular atrophy and extracellular matrix accumulation, always occurs in the early stage of DN and leads to chronic renal failure [2, 3]. However, the detailed mechanism of TIF is still unclarified.

Autophagy is a protective mechanism of eukaryotic cells that degrades protein aggregates, damaged organelles and other macromolecules in the cytoplasm [4]. Therefore, autophagy plays a prominent part for maintaining cell homeostasis [5]. The recent researches indicate that autophagy is generally suppressed in DN and the activation of autophagy is also found to be beneficial to resist T1D and prevent the progression of DN [6–8]. Although growing evidences confirm that autophagy can provide protection against fibrogenesis in DN, the event initiating this process remains unclear.

At present, there are more and more studies researching on relationship between endoplasmic reticulum stress (ERS) and autophagy [9, 10]. It is being increasingly recognized that ERS can also regulate autophagy [11]. Activating transcription factor 4 (ATF4), ERS related transcription factor, has a crucial role in the adaptation to stresses through modulating many physiological processes [12, 13]. Several studies suggested that the eIF α /ATF4 pathway plays a pivotal role in autophagy regulation. Song et al. demonstrated that intermittent hypoxia can protect pancreatic beta cell apoptosis by activating autophagy through endoplasmic reticulum stress-related signaling pathway PERK/ eIF2 α /ATF4 [14]. Bretin et al. demonstrated that activation of the EIF2AK4-EIF2A/eIF2 α -ATF4 pathway could trigger autophagy in response to Crohn disease-associated adherent-invasive *Escherichia coli* infection [15]. However, the role of ATF4 in modulation of autophagy in DN has not been explored.

This study focuses on the effect of ATF4 in regulation of autophagy in DN. We demonstrated that ATF4 inhibition suppressed renal tubulointerstitial fibrosis by restoring autophagy activity. It was concluded that ATF4 may serve as a potential therapeutic target in preventing the development of renal tubulointerstitial fibrosis in DN.

Material And Methods

Animals

Male ATF4^{+/-} heterozygous and ATF4^{+/+} wild-type (WT) mice in the C57BL/6J background were obtained from GemPharmatech Co., Ltd. All mice were raised in the environment of 21 ± 3 °C and light / dark cycle for 12 hours. Before the experiment, the mice need to adapt to the environment for at least one week, In the meantime, they are free to get water and food. All experiments were approved by the Ethics Committee on Animal Experiments of Jinan University (Approval No.2019022501). Fourteen WT and fourteen ATF4^{+/-} mice were randomly divided into control group (n = 7) and streptozotocin (STZ) group (n = 7) respectively. 150 mg/kg STZ (Sigma-Aldrich, St. Louis, USA) dissolved in 0.1 mM citrate buffer at pH 4.5 was administered to mice via an intraperitoneal injection to induce DN. Control group were injected with an equivalent volume of citrate buffer. When the blood glucose level is higher than 16.5 mmol/l, it is considered as a successful DN model. Ten weeks later, pentobarbital sodium (60 mg/kg iP) was used to anesthetize mice, and blood was collected from eyeballs to measure biochemical parameters. Renal tissues were collected for further study.

Cell Culture And Transfections

NRK-52E cells were purchased from The Cell Bank of Chinese Academy of Sciences. Cells were maintained in DMEM containing 5% fetal bovine serum with 5% CO₂. The temperature of the incubator is 37°C. To mimic normal and diabetic pathological conditions, cells were cultured in DMEM containing 5.5 mmol/L glucose (normal glucose, NG) or 30 mmol/L glucose (high glucose HG). pcDNA3.1A-ATF4 plasmid and vector were obtained from Hanbio Biotechnology Co., Ltd. (Shanghai, China). ATF4 siRNA and siRNA-NC vector were synthesized by Guangzhou Ribobio Co., Ltd. (GuangDong, China) In the experiment, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection.

Western blot analysis

After the total protein was extracted from renal tissue by RIPA method, BCA protein detection kit was used to determine the total protein content. Aliquots of proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes. After blocking, the membranes were blotted overnight with primary antibodies: anti-Col-IV (1:1000, Sigma), anti-LC3A/B (1:1000, CST), anti-P62 (1:2000, CST), anti-ATF4 (1:1000, CST), anti-GAPDH (1:1000, CST). After incubation with secondary antibodies (1:10000 dilution), chemiluminescence was performed with Pierce® ECL western blotting substrate. The gray value of bands was calculated by Gel-pro analyzer software.

Real-time Quantitative RT-PCR

Trizol kit (Invitrogen, Carlsbad, CA) were used to extract total RNA from collected samples. Then Invitrogen (Carlsbad, CA) kit were used to reverse transcribe RNA into cDNA for later PCR reaction. Quantitative RT-PCR was performed by bio rad 96fx circulatory (bio rad, USA) with SYBR Green Master Mix. The primer sequences of ATF4 gene and GAPDH reference gene are as follows: ATF4: forward 5'-CGACTTTTATTACACTTTCTGGGAG-3' and reverse 5'-GGGACAGATTGGATGTTGGAG-3'; GAPDH: forward 5'-TCTCTGCTCCTCCCTGTTC-3' and reverse 5'-ACACCGACCTTCACCATCT-3'. The results were analyzed by 2- $\Delta\Delta C_q$ method.

Transmission electron microscopy

Firstly, the samples were fixed with 2.5% glutaraldehyde and then fixed with 1% osmium tetroxide, embedded in the Durcupan ACM after dehydrated. Then uranyl acetate and lead citrate were used to stain ultrathin sections of samples. Finally, transmission electron microscopy (JEOL-100CXII, JEOL, Japan) was used to observe autophagosomes. Ten fields were randomly extracted from each group, and the number of autophagosomes was analyzed.

Tandem mRFP-GFP-LC3 Fluorescence Microscopy

The autophagy flow was observed by tandem mRFP-GFP-LC3 fluorescence microscopy. After 24 hours transfection of NRK-52E cells with adenovirus expressing mRFP-GFP-LC3 (Hanbio Co., Ltd., Shanghai, China), images were observed and collected by confocal laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany).

Histology and immunofluorescence

Harvested kidney samples were conventionally immersed in 4% paraformaldehyde overnight under ordinary temperature. 4 µm thick paraffin embedded mouse kidney sections were prepared. Hematoxylin and eosin (HE), Masson and Periodic acid-Schiff (PAS) staining were carried out and morphologic analysis was performed by light microscopy.

The expression of renal light chain 3 (LC3) was detected by immunofluorescence staining. The tissue sections were soaked with anti LC3B (1:200, CST) and incubated at 4 °C for about 12 hours, and then incubated with alexa Fluor 594-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG and cy3-conjugated goat anti-rabbit IgG at 37°C for 1 h. Finally, the nuclei were labeled with DAPI, and the images were observed and collected by laser scanning confocal fluorescence microscope (UltraVIEW VoX; PerkinElmer, Inc.).

Biochemical Assays

Detection of blood glucose in mice was performed with Glucometer (Roche Diabetes Care GmbH, UK). Serum creatinine and blood urea nitrogen was analyzed by an automatic chemistry analyzer (AU480; Beckman Coulter Inc; Kraemer Boulevard Brea, CA). Mice stayed in a metabolic cage and urine was collected for 24 hours. The level of urinary albumin was determined by ELISA Kit (Bethel laboratory, Montgomery, TX).

Statistical analysis

All experimental data are shown as mean ± SE. SPSS software 22.0 is used for statistical analysis (SPSS, Inc.). When two groups are compared, Student's t-test is selected for analysis. One-way ANOVA with Tukey post hoc test was performed to analysis when comparing three or more groups. The results were considered to be significant with values of $P < 0.05$.

Results

Expression of ATF4 in the kidneys of DN mice and HG-treated NRK-52E cells.

STZ-induced DN in WT mice had markedly decreased weight and increased fasting blood glucose, urinary albumin, serum creatinine and blood urea nitrogen levels compared with WT-Control group (Table 1). In order to determine the expression of ATF4 in DN mice, western blotting and RT-PCR analysis was

conducted, and the results showed that the expression of ATF4 was upregulated in DN mice compared with that in the control mice (Fig. 1A and B). Moreover, we validated the expression level of ATF4 in vitro. The results showed that the expression of ATF4 in NRK-52E cells cultured in HG condition was upregulated than cells cultured in NG condition (Fig. 1C and D). Therefore, we confirmed that the expression of ATF4 was significantly upregulated in both DN mice and NRK-52E cells after high glucose intervention.

Table 1

Physical and biochemical parameters of the experimental animals (Data were expressed as mean ± SE)

Variable	Control		STZ	
	WT	<i>Atf4</i> ^{+/-}	WT	<i>Atf4</i> ^{+/-}
Body weight (g)				
0 Weeks	18.7 ± 0.3	19.4 ± 0.3	19.2 ± 0.3	19.3 ± 0.4
10 Weeks	22.2 ± 0.7	22.5 ± 0.6	17.1 ± 0.5 ^a	17.6 ± 0.7 ^a
Fasting blood glucose (mmol/l)	7.5 ± 0.3	7.0 ± 0.2	23.0 ± 1.3 ^a	22.6 ± 1.2 ^a
Urinary albumin (µg/24 h)	50.4 ± 2.5	47.3 ± 3.4	472 ± 13.9 ^a	411.4 ± 10.6 ^{ab}
Serum creatinine (µmol/l)	31.8 ± 2.0	26.7 ± 1.9	73.2 ± 3.6 ^a	58.6 ± 3.5 ^{ab}
Blood urea nitrogen (mmol/l)	8.5 ± 0.6	7.4 ± 0.7	16.9 ± 1.2 ^a	15.5 ± 1.0 ^a
STZ: streptozotocin. WT: wild type. ^a <i>P</i> <0.05 versus Control WT group; ^b <i>P</i> <0.05 versus STZ WT group.				

Autophagy and fibrosis levels in STZ-induced DN mice and HG-treated NRK-52E cells.

To identify the role of autophagy in DN, we detected the expression of autophagy-related proteins. The results indicated that the protein expression of p62 increased and the ratio of LC3-II/ LC3-I decreased markedly in the kidneys of STZ group than that of the control group (Fig. 2A). Similarly, we also observed an increase of p62 and a decreased ratio of LC3-II/ LC3-I in NRK-52E cells cultured in high glucose condition (Fig. 2B). Furthermore, the level of Col-IV was elevated in NRK-52E cells after high glucose intervention compared with that cultured in normal glucose condition (Fig. 2B). In addition, we observed the changes of autophagic vesicles of NRK-52E cells after treatment with HG by transmission electron microscope. Autophagosomes count was performed on 10 randomly selected regions in each group. The number of autophagosomes was evidently decreased in cells cultured in high glucose condition compared with that cultured in normal glucose condition (Fig. 2C). Our results suggested that autophagy was suppressed and fibrosis was deteriorated in DN.

ATF4 inhibited autophagy and promoted fibrosis in HG-treated NRK-52E cells.

To confirm whether ATF4 regulates autophagy in NRK-52E cells cultured in high glucose condition, the levels of autophagy-related proteins was detected by western blotting. The expression of p62 protein was increased markedly in cells transfected by pcDNA3.1A-ATF4 plasmid than that transfected by vector under HG conditions, while decreased markedly in cells transfected by siATF4 than that transfected by siNC under HG conditions (Fig. 3). In addition, gene silencing of ATF4 restored the levels of LC3- β protein in NRK-52E cells cultured in HG condition (Fig. 3). We also demonstrated that siATF4 could reduce the expression of Col-IV compared with the siNC group with HG treatment (Fig. 3). We further examined the autophagosomes in NRK-52E cells through transmission electron microscopy. We found that the number of autophagosomes was increased in siATF4-transfected cells group compared with the siNC-transfected cells group under HG treatment (Fig. 4A). Finally, we utilized the tandem GFP-RFP-LC3 to observe the autophagy flux. As shown in Fig. 4B, we observed less red puncta in the control group with high glucose treatment than that in the control group with normal glucose treatment. After the HG-induced cells transfected by siATF4, the numbers of red puncta increased than that transfected by siNC, which indicated that inhibition of ATF4 could restore autophagosomes and autolysosomes. These data suggested that ATF4 aggravate fibrosis by inhibiting autophagy.

Genetic inhibition of ATF4 ameliorates renal fibrosis in STZ-induced DN mice

STZ was injected into heterozygous ATF4 KO mice and WT mice to induce DN. We used heterozygous ATF4 mice because previous studies reported that majority of the homozygous mice were neonatal lethal and surviving mice were dwarf [16, 17]. Our results showed that there were no differences in blood glucose and body weight between WT-STZ and ATF4 KO-STZ group (Table 1). However, ATF4 KO-STZ mice showed markedly decreased urinary albumin, serum creatinine and blood urea nitrogen compared with WT-STZ mice (Table 1).

We next determined if ATF4 inhibition ameliorates retinal fibrosis in DN mice. Compared with WT-Control group, WT-STZ group showed increase in mesangial matrix expansion and glomerular volume, mesangial cell proliferation, basement membrane thickening and significant renal fibrils accumulation (Fig. 5). Pathologic injury of the kidney tissues was markedly improved in ATF4 KO-STZ group compared with WT-STZ group.

Genetic inhibition of ATF4 restores autophagy in STZ-induced DN mice

Finally, we determined whether ATF4 had an effect on autophagy in DN mice. Western blot showed that ATF4 KO-control mice had significantly decreased expression of ATF4 compared with WT-control mice, which indicated that heterozygous ATF4 KO mice was suitable to be used in the present study. Compared with WT-Control group, WT-STZ group had significantly increased protein level of p62 and decreased ratio of Lc3- β / Lc3- α (Fig. 6A and B). ATF4 KO-STZ mice showed markedly downregulated protein level of p62 and upregulated ratio of Lc3- β / Lc3- α , which suggested that genetic inhibition of ATF4 can regulate expression of autophagy-related proteins. Furthermore, we also detected the levels of autophagic activity marker by immunofluorescence staining. The LC3B fluorescence area was markedly decreased in WT-STZ

group than that in WT-control group. Genetic inhibition of ATF4 can increase the LC3B fluorescence area (Fig. 6C).

Discussion

We demonstrated that ATF4 can inhibit autophagy and promote the expression of Col-IV in NRK-52E cells cultured in high glucose condition and genetic inhibition of ATF4 can ameliorate renal tubulointerstitial fibrosis by restoring autophagy in STZ-induced DN mice. Our study provided the first evidence of a crucial role for ATF4 in modulating autophagy against diabetic kidney damage. Suppression of ATF4 may be an effective therapy in restraining renal tubulointerstitial fibrosis.

Our data suggested that the regulation of autophagy may be a key part in treating renal interstitial fibrosis in DN. Some studies have demonstrated that the activity of autophagy was generally downregulated in kidney tissues of DN and the activation of autophagy was also found to be beneficial to resist the cell damage induced by high glucose in vitro [18–20]. It indicates that the induction of autophagy may provide protection against DN. At present, more and more studies have confirmed that tubular autophagy is closely related to the occurrence of tubulointerstitial fibrosis [21, 22]. Zhang et al. found that miR-22 may promote renal tubulointerstitial fibrosis by inhibiting autophagy in NRK-52E cells partially via targeting PTEN [23]. Liu et al. found that Notch 1 can regulate the expression of PTEN by suppressing autophagy of NRK-52E cells, which led to tubulointerstitial fibrosis in diabetic nephropathy [24]. Our findings revealed a declined number of autophagosomes in NRK-52E cells after treatment with high-glucose. In addition, western blotting assays showed downregulated expression of LC3 and upregulated expression of p62 protein in DN mice and high glucose-treated NRK-52E cells. Furthermore, Col-IV was increased in NRK-52E cells cultured in high glucose condition and DN mice showed severe renal tubulointerstitial fibrosis. These results implied that the regulation of autophagy has an important role in the treatment of renal tubulointerstitial fibrosis in DN.

Recent research have indicated that ERS plays a vital role in regulating autophagy [25, 26]. Studies have shown that ERS can activate or inhibit autophagy in different situations [27]. ATF4 is a downstream effector of PERK-UPR branch in ERS, which is closely related to renal tubular injury in DN [28–31]. Our results showed that expression of ATF4 was evidently upregulated in both HG-treated NRK-52E cells and kidney tissues of DN mice. The relationship between ATF4 and autophagy in DN has not been reported yet. To elucidate the function of ATF4 in regulation of autophagy in DN, we overexpressed and inhibited ATF4 in the NRK-52E cells. The results showed that the transfection of pATF4 plasmid could inhibit autophagy and upregulate the level of Col-IV. On the contrary, inhibition of ATF4 could increase the number of autophagosomes, promote autophagy flux and reduce Col-IV in high glucose induced NRK-52E cells. Then we utilized heterozygous ATF4 KO mice to explore the regulation of ATF4 on autophagy in vivo. Our results showed that genetic inhibition of ATF4 did not reduce the blood glucose significantly in DN mice, which is similarly to the previous study [32]. However, downregulation of ATF4 can improve urinary albumin, serum creatinine and blood urea nitrogen and the pathological changes of renal tubulointerstitial fibrosis. Furthermore, western blotting assays and immunofluorescence staining

revealed that inhibition of ATF4 could restore autophagy in DN mice. We found that ATF4 had an inhibit effect on autophagy in DN, which is opposite to the previous studies showing that downregulation of ATF4 can inhibit autophagy in brain tumor initiating cells [33]. We speculate that the different function of ATF4 on autophagy may be related to different cells.

Conclusion

In summary, we confirmed that ATF4 had an important effect on regulation of renal tubulointerstitial fibrosis in DN by inhibiting autophagy, which had not been shown before. These results indicate that targeting ATF4 may be a potential therapy in preventing the development of renal tubulointerstitial fibrosis in DN.

Abbreviations

DN: diabetic nephropathy; ATF4: activating transcription factor 4; STZ: streptozotocin; KO: knockout; WT: wild-type; TIF: tubule interstitial fibrosis; ERS: endoplasmic reticulum stress; NG: normal glucose; HG: high glucose; HE: Hematoxylin and eosin; PAS: Periodic acid-Schiff; LC3: renal light chain 3

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors read and approved the final manuscript.

Availability of data and materials

The data sets used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors confirm that there are no competing interests.

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Authors' contributions

YX and LC designed the study; QL and TL conducted the experiments and obtained the data; WT, XC and WC analyzed and collated the data; QL, TL, and TG drafted the manuscript.

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Figures

Figure 1

Expression of ATF4 in the kidneys of STZ-induced DN mice and HG-treated NRK-52E cells. (A) The proteins expression of ATF4 in STZ-induced DN mice detected by Western blot. (B) The mRNA expression of ATF4 in STZ-induced DN mice detected by real-time PCR. *P < 0.05 versus Control group. (C) The proteins expression of ATF4 in HG-treated NRK-52E cells detected by Western blot. (D) The mRNA expression of ATF4 in HG-treated NRK-52E cells detected by real-time RT-PCR. STZ: streptozotocin, NG: normal glucose, HG: high glucose. *P < 0.05 versus NG group.

Figure 2

Autophagy and fibrosis levels in STZ-induced DN mice and HG-treated NRK-52E cells. (A) The expression of autophagy-related proteins in STZ-induced DN mice. STZ: streptozotocin. * $P < 0.05$ versus Control group. (B) The expression of Col-IV and autophagy-related proteins in HG-treated NRK-52E cells. (C) Representative electronic micrographs and summarized data showing the number of autophagosomes/well in different groups (Autophagosomes was counted in 10 randomly selected fields). Autophagic vacuoles were indicated with arrows. NG: normal glucose, HG: high glucose. * $P < 0.05$ versus NG group.

Figure 3

ATF4 regulates the expression of autophagy and Col-IV in HG-treated NRK-52E cells. (A-C) The expression of protein p62, LC3-I, LC3-II and Col-IV were observed by Western blotting after overexpression and inhibition of ATF4. pATF4: pcDNA3.1A-ATF4 plasmid group; p-Ctrl: plasmid vector group; SiATF4: ATF4 small interfering RNA group; Si-Ctrl: siRNA negative control group. HG: high glucose. * $P < 0.05$ versus p-Ctrl group; # $P < 0.05$ versus Si-Ctrl group; ▲ $P < 0.05$ versus p-Ctrl group in HG condition; ▼ $P < 0.05$ versus Si-Ctrl group in HG condition.

Figure 4

ATF4 inhibits autophagic vesicles and autophagic flow in HG-treated NRK-52E cells. (A) Observation of autophagic vesicle formation by transmission electron microscopy (Autophagosomes was counted in 10 randomly selected fields). Autophagic vesicles were labeled by arrows. NG: low glucose; HG: high glucose. *P < 0.05 versus NG-Control group; #P < 0.05 versus HG-Control group. (B) Detection of autophagy flow by mRFP-GFP-LC3 tandem fluorescent protein quenching assay. NG: low glucose; HG: high glucose.

Figure 5

Genetic inhibition of ATF4 ameliorates renal fibrosis in STZ-induced DN mice. The pathologic changes of renal tissues were measured via HE, PAS and Masson staining. (magnification, $\times 400$). STZ: streptozotocin. WT: wild type. KO: heterozygous ATF4 KO mice.

Figure 6

Genetic inhibition of ATF4 restores autophagy in STZ-induced DN mice. (A) The expression of protein P62, LC3 α , LC3 β and ATF4 were observed by Western blotting. (B) The expression of LC3B was determined via immunofluorescence. STZ: streptozotocin group. STZ: streptozotocin. WT: wild type. KO: heterozygous ATF4 KO mice. *P < 0.05 versus WT-Control group; #P < 0.05 versus WT-STZ group.