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Notch1 regulates PTEN expression to exacerbate renal tubulointerstitial fibrosis in diabetic nephropathy by inhibiting autophagy via interactions with Hes1

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ABSTRACT

Diabetic nephropathy (DN) is a serious clinical microvascular complication of diabetes mellitus. DN is characterized by the accumulation of extracellular matrix, resulting in progressive fibrosis leading to the loss of renal function. Notch1 and phosphatase and tensin homolog deleted on chromosome ten (PTEN) signaling have been associated with fibrosis. Autophagy serves as an essential regulator of tubular cellular homeostasis. However, how these molecules control the balance between fibrosis and autophagy, the main homeostatic mechanism regulating fibrosis, is not well understood. This association was confirmed using Notch1-siRNA in vitro, which prevented the increase in Hes1 and restored PTEN expression. In contrast, transfection with pHAGE-Hes1 repressed PTEN promoter-driven luciferase activity, implying a direct relationship between Hes1 and PTEN. The expression of Notch1 and Hes1 was increased in diabetic db/db mice by western blotting; in contrast, the expression of PTEN was decreased. Importantly, the dysregulation of these signaling molecules was associated with an increase in extracellular matrix proteins (Collagen-I and III) and the inhibition of autophagy. Similar results were evident in response to high glucose concentrations in vitro in the NRK-52e cells. Therefore, the high glucose concentrations present in diabetes promote fibrosis through the Notch1 pathway via Hes1, while inhibiting the PTEN and autophagy. In conclusion, the inhibition of PTEN by Notch1/Hes1 in response to high glucose concentration inhibits autophagy, which is associated with the progression of fibrosis. Therefore, these signaling molecules may represent novel therapeutic targets in diabetic nephropathy.

1. Introduction

Diabetic nephropathy (DN) is the major cause of end-stage renal disease (ESRD) [1]. Progressive renal tubulointerstitial fibrosis occurs in virtually every type of chronic kidney disease. DN is characterized by the accumulation of extracellular matrix (ECM), resulting in progressive kidney fibrosis that leads to kidney function decline and irreversible loss of tissue [2]. The increasing prevalence of interstitial fibrosis in diabetic nephropathy is why this disease has been one of the current focuses of research.

Autophagy is a lysosomal protein degradation pathway and efficient intracellular protein degradation system essential for maintaining cellular homeostasis [3]. Autophagy plays an important role in kidney injury [4,5]. Growing evidence reveals that the impairment of autophagy in the kidney is associated with the pathogenesis of DN, and autophagy restoration may be renoprotective [6,7]. Importantly, basal autophagy activity in tubular cells is presumably low under normal physiological conditions [8]. Autophagy induction in proximal tubular cells, plays an important role in protecting cells [9]. The inhibition of autophagy in tubular cells exacerbates fibrogenesis in DN. Other autophagy inducers and mTORC1 interactors has been likely suppressed in the development of DN [10,11]. How precisely autophagy executes its protective role in renal tubular cells undergoing injury remains unclear.

However, the mTOR signaling pathway is regulated by

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numerous other upstream signaling pathways, including PI3K/Akt, AMPK, and p53. In particular, the mTOR pathway is activated when cellular energy crisis, and in this manner, modulates autophagy based on the cellular energy requirements [12]. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a key regulator of AKT/mTOR signaling. PTEN is a tumor suppressor that is lost or mutated in various malignancies [13]. Recent studies suggest a role for PTEN in the progression of tissue fibrosis, such as the liver, lung and kidneys, by activating profibrotic signaling pathways [14–16]. PTEN loss in tubular cells initiates a series of events resulting in epithelial dedifferentiation, the expression of a subset of fibrotic factors and growth arrest. This finding suggests the loss of PTEN expression by some unknown mechanism(s) during renal fibrosis. Some micro-RNAs, including miR-22, miR-214 and miR-217, can directly target PTEN expression [17–19]. Additionally, PTEN may be regulated by other factors, such as Notch signaling, which inhibits the activity of PTEN, indicating the importance of the inhibition of Notch signaling in gastric cancer therapy [20]. However, the precise mechanisms of how Notch1 affects the loss of PTEN expression during renal injury are not clear. In the present study, we explored the role of Notch1 and PTEN in autophagy and fibrosis, along with the possible mechanism of renal tubulointerstitial fibrosis in DN. We speculated that PTEN could restore autophagy to alleviate fibrosis via the Notch1/PTEN pathway, resulting in the improvement of renal tubulointerstitial fibrosis in DN.

2. Materials and methods

2.1. Animals

All the animal experimental procedures were reviewed and approved by the Ethics Committee of the Guizhou Medical University. The db/db (approximately 6 weeks old) male mice and db/m mice were supplied by the Nanjing Center for Experimental Animals (Nanjing, China). The conditions were controlled as follows: temperature (20–25 °C), relative humidity (50–60%). The mice were randomly assigned to two groups (8 mice per group): 1) db/m control group, and 2) db/db experimental group (8 mice per group). All mice were provided normal diets and unlimited drinking water. After ten weeks, eyeball blood was collected from each mouse prior to anesthetization with intraperitoneal sodium pentobarbital (50 mg/kg). Blood samples were taken for measuring biochemical parameters, and kidneys were collected for histological examination and molecular assays.

2.2. Cell culture and transfections

The NRK-52e cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. The pHAGE-Hes1 plasmid and vector were purchased from Era Biotech (Shanghai, China). The p pEF.hICN1.CMV.GFP-Notch1 plasmid was purchased from Addgene (USA). siRNA was designed and synthesized by Guangzhou Ribobio Co. Ltd. (GuangDong, China). All transfections were performed using Lipofectamine™ 3000 reagent (Invitrogen, USA). The cells were harvested at 48 h after transfection.

2.3. Western blotting analysis

After the proteins were isolated from the kidney tissues or cells and transferred to membranes, and the membranes were incubated overnight at 4 °C with rabbit anti-rat Notch1 (1:1000, CST), anti-Hes1 (1:1000, Abcam), anti-PTEN (1:1000, Abcam), anti-LC3 (1:1000, CST), anti-P62 (1:1000, Abcam), anti-Collagen-I (1:1000, Proteintech) and anti-Collagen-III (1:800, Sigma) monoclonal antibodies, as well as mouse anti-β-actin (1:4000, Biological Pumei, China). The membranes were repeatedly washed, and then subsequently incubated with secondary antibodies for 1 h. Clarity ™ Western ECL substrate (BIO-RAD, USA) was added to visualize the proteins.

2.4. RNA extraction and quantitative RT-PCR

Total RNA was extracted from the kidney tissues or cells. Then, the cDNA was synthesized using the cDNA Synthesis Kit (Takara). Subsequently, the SYBR Premix Ex TaqTM II Kit (Takara) was used to perform the RT-PCR assay. The quantification of gene expression was standardized to the β-actin mRNA levels. The data were processed using the $2^{-\Delta\Delta CT}$ method.

2.5. Immunohistochemistry and immunofluorescence

After antigen retrieval and blocking, the sections were incubated at room temperature with an optimal dilution of Notch1, Hes1, PTEN, LC3, Collagen-I and III monoclonal antibodies for 24 h. After anti-rabbit secondary antibody was incubated and developed with DAB and counterstained with hematoxylin. The NRK-52e cells fixed at 4 °C in 4% paraformaldehyde. The cells were exposed to antibodies against Notch1, PTEN, Hes1, Collagen-I and III overnight. The cells were incubated with the CY3 goat anti-rabbit IgG (H + L) (red) (Proteintech, USA) at 37 °C for 1 h. The fluorescence was observed under a fluorescence microscope (Leica, DM4000B, Germany).

2.6. Confocal microscopy analysis

The cells were transfected with mRFP-GFP-LC3 adenovirus. Sixteen hours after transfection, the cells were re-transfected with siNotch1 for 48 h. Positive controls were treated with the autophagy inducer, rapamycin, at 100 nM for 48 h. Subsequently, the cells were fixed, stained with DAPI to dye the cell nucleus and immediately analyzed by confocal microscopy (Olympus FV1000, Japan).

2.7. Dual-luciferase reporter assay

pGl3-PTEN-promoter luciferase reporter plasmids containing the PTEN promoter region were constructed by Era Biotech (Shanghai, China).The cells were plated onto 24-well plates. The lysates were collected at 48 h after transfection, and luciferase activity was measured with the dual luciferase assay (Promega).

2.8. Statistical analysis

Statistical analysis was performed using SPSS software, version 19.0. The data are expressed as the means ± standard deviation (SD). The statistical significance of differences was calculated using the *t*-test analysis of variance (ANOVA), and $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Activation of the Notch signaling in vivo and in vitro

To investigate the target of the Notch signaling in diabetic mice, we performed western blotting analysis, and the results indicated that the protein expression of Notch1 and Hes1 was increased in db/db group compared with that in the db/m group (Fig. 1A and B). Relative to the db/m group, the mRNA levels of Notch1 and Hes1 were increased in the renal tubulointerstitium of the db/db group

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