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Nrf2 ameliorates diabetic nephropathy progression by transcriptional repression of TGFβ1 through interactions with c-Jun and SP1

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ABSTRACT

Diabetic nephropathy (DN) is one of the major complications in diabetes patients. Reactive oxygen species (ROS) play key roles in DN progression. As a primary transcription factor, Nrf2 controls the antioxidant response to maintain cellular redox homeostasis. Herein we systemically examined the role of Nrf2 in DN progression and its regulatory mechanism in a mouse model bearing type II diabetes and in cultured human renal mesangial cells (HRMCs). We found that Nrf2 could ameliorate DN progression by transcriptional repression of TGFβ1 *in vivo* and *in vitro*. Moreover, Nrf2 bound to the specific region in *TGFβ1* promoter by interactions with transcription factors c-Jun and SP1. Significant abolishment of Nrf2-mediated *TGFβ1* transcriptional repression could be accomplished by knockdown of either *c-Jun* or *SP1*, and site-directed mutagenesis of c-Jun and SP1 binding sites in the *TGFβ1* promoter specific region. Moreover, after interacting with c-Jun and SP1, Nrf2 inhibited c-Jun and SP1 activations, and thus reversed c-Jun- and SP1-promoted *TGFβ1* transcription. In all, Nrf2 could slow down DN progression by repression of *TGFβ1* in a c-Jun and SP1-dependent way. Our findings may provide novel clues for DN preventions and interventions in clinic.

1. Introduction

Diabetes mellitus (DM) is a multisystem disorder that affects various organs. Almost 30% of DM patients develop diabetic nephropathy (DN), which is the leading cause of end-stage renal disease (ESRD) worldwide [1,2]. The annual incidence of diabetic nephropathy is increasing with the greatest prevalence found in America, Japan and industrialized European countries [3,4]. Diabetic nephropathy is clinically characterized by proteinuria accompanied by decreased glomerular filtration rate (GFR) [5], and histologically defined by glomerular basement membrane (GBM) thickening and mesangial matrix expansion from the accumulation of extracellular matrix (ECM) proteins [6,7]. The main feature of DN is excessive production of reactive oxygen species (ROS) which can induce renal damage under continuous hyperglycemic conditions [8]. In response to hyperglycemia, many renal cell types, including mesangial cells, endothelial cells and tubular epithelial cells were reported to produce high levels of ROS [9–12]. Overproduced ROS in the kidney directly oxidize and damage macromolecules, including DNA, lipid, protein, and carbohydrate [13]. Additionally, ROS also function as signal molecules to mediate hyperglycemia-induced activation of signal transduction cascades and transcriptional factors leading to transcriptional activation of profibrotic genes (*e.g.* transforming growth factor β1 (TGFβ1)) [14]. In turn, inhibition of ROS prevents advanced glycation end product-mediated renal damage in DN [11]. Antioxidants have always been shown to blunt oxidative stress in the glomeruli of diabetic mice [12]. Therefore, antioxidant therapies may be able to prevent or treat DN.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is the primary transcription factor that controls the antioxidant response to maintain

Abbreviations: DM, diabetes mellitus; DN, diabetic nephropathy; ESDR, end-stage renal disease; GFR, glomerular filtration rate; GBM, glomerular basement membrane; ECM, extracellular matrix; ROS, reactive oxygen species; Nrf2, nuclear factor erythroid 2-related factor 2; KEAP1, kelch-like erythroid cell derived protein with CNC homology (ECH)-associated protein 1; ARE, antioxidant-responsive elements; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; TGFβ1, transforming growth factor-β1; FN, fibronectin; NQO1, NADPH quinine oxidoreductase-1; SP1, specificity protein 1; UACR, urine albumin to creatinine ratio; NAC, N-acetylcysteine; SFN, sulforaphane; HE, hematoxylin-eosin; PAS, periodic acid–schiff stain

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cellular redox homeostasis [15,16]. Upon exposure of cells to oxidative stress or electrophilic compounds, Nrf2 dissociates from kelch-like erythroid cell derived protein with CNC homology (ECH)-associated protein 1(KEAP1) and translocates into the nucleus to bind to antioxidant-responsive elements (ARE) in gene promoters, encoding for antioxidant enzymes and phase II detoxifying enzymes, including NAD(P)H quinone oxidoreductase (NQO1), heme oxygenase-1 (HO-1), glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), and γ-glutamylcysteine synthetase (γ-GCS). Up-regulation of these Nrf2-dependent genes protects against insults such as oxidative stress and inflammation that are known to be enhanced by the diabetic milieu [17,18]. To date, mounting evidence indicates that Nrf2 plays critical roles in the prevention of diabetic complications, including diabetic nephropathy [16]*via* Nrf2-mediated antioxidant pathway. It was shown that $Nrf2^{-/-}$ mice suffered severer streptozotocin-induced renal damage in contrast to $Nrf2^{+/+}$ mice [16]. A recent study has shown that Nrf2 activation by sulforaphane (SFN) reduced oxidative damage in streptozotocin-induced diabetic nephropathy [19]. Activation of Nrf2 by SFN also suppressed hyperglycemia-induced ROS in human microvascular endothelial cells [20] and in a type I diabetic mouse model [17]. The activation of Nrf2-mediated antioxidant pathway was even closely linked with vitamin D-ameliorated DN progression [4]. In fact, Nrf2-mediated transcriptional responses are protective in a variety of experimental animal models, including streptozotocin-induced diabetic nephropathy, oxidative lung injury and fibrosis, asthma, and brain ischemia–reperfusion damage [16,21–23]. All these studies have indicated the critical roles of Nrf2 and its activators in DN progression.

During DN progression, TGFβ1 overexpression, ECM deposition and loss of glomerular architecture define glomerulosclerosis [12]. Accumulated evidence suggested that TGFβ1 plays a central role in diabetic nephropathy progression by regulating expressions of fibronectin (FN) and type IV collagen which are the main components of ECM [24–26]. Interestingly, Nrf2 could also regulate ECM production and thus confer protection against fibrosis. For example, activation of Nrf2 is able to inhibit the function of TGFβ1 in a liver fibrosis model [27] and a streptozotocin-induced diabetic nephropathy model [16]. However, the mechanism by which Nrf2 down-regulates expression of TGFβ1 remains unclear.

Since DN is prevalent in patients with type II DM of many ethnicities, including African American, Hispanic and Asian [1], a type II DM mouse model was used. We systematically examined the role of Nrf2 in DN progression, as well as the mechanism of how Nrf2 negatively regulates TGFβ1 to control ECM production in ameliorating DN progression.

2. Materials and methods

2.1. Animals and treatments

Five-week-old C57BLKS/J-Lepr (db) (db/db) mice genetically bearing type II DM were purchased from the Model Animal Research Center of Nanjing University, Nanjing, China, and evenly divided into a SFN (Santa Cruz, CA, USA)-treated group and a vehicle-treated group ($n = 10$ for each group). The *db/db* mouse (in C57BLKs/J background) model used here is a well established type II DM model that develops obesity spontaneously, hyperinsulinemia at 10 days of age, and slight hyperglycemia at 1 month of age. Overt hyperglycemia is noted by 8 weeks of age [28,29]. Albuminuria can be detected 3 to 4 weeks after the presence of hyperglycemia [30]. Glomerular changes, such as hypertrophy and mesangial matrix expansion display by 5 to 6 months of age [28]. Ten normal C57BLKS/J mice (five-week-old) were used as control. Mice from three groups (control, DM, and DM + SFN group, respectively) were housed with *ad libitum* access to food and water in light and temperature-controlled environments. All mice were treated with the same amount of PBS. In addition, the DM + SFN group mice were intraperitoneally injected with SFN (12.5 mg/kg) 3 times per week. Glucose level and body weight were monitored once every two weeks for a period of 15 weeks. Urine albumin to creatinine ratio (UACR) was also measured at age 9, 16 and 23 weeks. At age 23 weeks, all mice were sacrificed and both kidneys were dissected for subsequent analysis. The protocol was approved by the Institutional Animal Care and Use Committee of Fudan University (permission number: 20120302-049), and all efforts were made to minimize suffering.

2.2. Histology and immunohistochemistry (IHC) analysis

On dissection, both kidneys were isolated and weighed. Tissues were cut into 4 μm sections and initially stained with hematoxylin-eosin (H&E), periodic acid–schiff (PAS) and Masson's trichrome, respectively. Paraffin-embedded sections were then subjected to antigen retrieval in a microwave in 0.1 M citric acid buffer (PH 6.0), and thereafter incubated with corresponding primary antibodies at 4 °C overnight. After secondary antibody incubation at room temperature for 1 h, slides were developed in 0.05% diaminobenzidine containing 0.01% H_2O_2. For negative controls, specific antibodies were replaced with normal goat serum by co-incubation at 4 °C overnight preceding IHC staining procedure.

2.3. Cell culture and antibodies

Human renal mesangial cells (HRMCs) were purchased from ScienCell (San Diego, CA, USA) and cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Los Angeles, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco). In some experiments, HRMCs were also cultured in DMEM with low glucose (5.6 mmol/l), low glucose plus N-acetylcysteine (NAC) (Sigma, St. Louis, MO, USA), high glucose (30 mmol/l), and high glucose plus NAC before harvest. Primary antibodies against Nrf2, NQO1 and GAPDH and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against TGFβ1 (Abcam, Cambridge, UK) and FN (Calbiochem, CA, USA) were purchased from commercial sources. c-Jun and SP1 antibodies were purchased from Cell Signaling (Boston, MA, USA).

Prior to tests, HRMCs stably overexpressing and silencing Nrf2 were also constructed. The negative control small interference RNA (siNC, 5′-UUCUCCGAACGUGUCACGUTT-3′), and Nrf2 siRNA (5′-CAUUGAUGUUUCUGAUCUATT-3′) were designed by Qiagen (Shanghai, China). Sequences were synthesized and inserted into the LV3-GFP vector (named as LV3-siNC or LV3-siNrf2). For Nrf2 overexpression, the Nrf2 expression plasmid was cloned into LV5-GFP vector (named as LV5-Nrf2). Thereafter, HEK 293T cells (1×10^6 cells) were transfected with the respective plasmid (LV3-siNC, or LV3-siNrf2, or LV5-NC, or LV5-Nrf2) and packaging vectors by GenePharma Co. Ltd (Shanghai, China). Lentiviral particles (LV3-siNC, or LV3-siNrf2, or LV5-NC, or LV5-Nrf2) were harvested 48 h post-transfection and purified by ultra centrifugation. HRMCs were then infected with the packaged lentivirus under medium culture. The viral titer was determined by counting green fluorescent protein-expressing cells under fluorescence microscopy 96 h after infection. HRMCs stably expressing Nrf2 and silencing Nrf2 were screened by puromycin (5 μg/ml).

2.4. qRT-PCR and immunoblot assay

Total RNAs of kidney tissues or HRMCs were extracted using Trizol solution (TaKaRa, Shiga, Japan) at 48 h after transfection. Fast-strand cDNA was generated from 1 μg of total RNAs using the PrimeScript RT Master Mix Perfect Real Time (TaKaRa, Shiga, Japan). Real-time quantitative PCR was then performed in an ABI PRISM 7900 Real-Time System using the SYBR Premix Ex Taq™ Perfect Real Time (TaKaRa, Shiga, Japan). For animal analysis, RNA lysates of ten mouse kidneys from the same group were extracted and pooled together to get total RNAs. Mean values and standard deviations for each group were obtained from triplicate analysis of the same pooled total RNAs. The primers used here are presented in Table 1. Relative mRNA levels were normalized to GAPDH. For the immunoblot assay, protein lysates from ten mice