The protective role of Nrf2 in STZ-induced diabetic nephropathy

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Objective: Diabetic nephropathy (DN) is one of the major causes of renal failure, which is accompanied by production of reactive oxygen species (ROS). Nrf2 is the primary transcription factor that controls the antioxidant response essential for maintaining cellular redox homeostasis. Here, we report our findings demonstrating a protective role of Nrf2 against DN.

Research design and methods: We explore the protective role of Nrf2 against DN using human kidney biopsy tissues from DN patients, a streptozotocin (STZ)-induced DN model in Nrf2/- mice, and cultured human mesangial cells (HRMCs).

Results: The glomeruli of human DN patients were under oxidative stress and had elevated Nrf2 levels. In the animal study, Nrf2 was demonstrated to be crucial in ameliorating STZ-induced renal damage. This is evident by Nrf2/- mice having higher ROS production and suffering from greater oxidative DNA damage and renal injury, compared to Nrf2/+ mice. Mechanistic studies in both in vivo and in vitro systems showed that the Nrf2-mediated protection against DN is, at least, partially through inhibition of transforming growth factor-β1 (TGF-β1) and reduction of extracellular matrix (ECM) production. In human renal mesangial cells, high glucose induced ROS production, and activated expression of Nrf2 and its downstream genes. Furthermore, activation or overexpression of Nrf2 inhibited the promoter activity of TGF-β1 in a dose-dependent manner, whereas knockdown of Nrf2 by siRNA enhanced TGF-β1 transcription and FN production.

Conclusions: This work clearly indicates a protective role of Nrf2 in DN, suggesting that dietary or therapeutic activation of Nrf2 could be used as a strategy to prevent or slow down the progression of DN.
Among the various types of diabetes mellitus, DN is the most common renal complications and the leading cause of end-stage renal diseases. The prevalence of diabetes is high in the United States, Japan, and most industrialized European countries (1). As a chronic disease, DN is characterized by sequential pathological changes, including renal hypertrophy and basement membrane thickening in the early stage, and ECM accumulation, glomerulosclerosis, and interstitial fibrosis in the late stage, which eventually results in the loss of renal function (2; 3). Although the pathogenesis of DN is complex and remains unclear, hyperglycemia is the primary factor that underlies the initiation of DN (4). It has been demonstrated in several in vitro studies that high glucose-induced renal damage is associated with excessive production of ROS under hyperglycemic conditions (4-6). In supporting this notion, many renal cell types including mesangial cells, endothelial cells, and tubular epithelial cells, were found to produce high levels of ROS under hyperglycemic conditions (7-10).

Nrf2 is one of the most important cellular defense mechanisms to cope with oxidative stress (11; 12). It regulates intracellular antioxidants, phase II detoxifying enzymes, and many other proteins that detoxify xenobiotics and neutralize ROS to promote cell survival and maintain cellular redox homeostasis (13). NAD(P)H quinone oxidoreductase (NQO1), glutathione S-transferase (GST), heme oxygenase-1 (HO-1), and \(\gamma\)-glutamylcysteine synthetase (\(\gamma\)GCS), are among the well-studied Nrf2 target genes that are upregulated through the antioxidant response element (ARE) regulatory element in response to oxidative stress (14; 15). The essential role of Nrf2 in combating oxidative stress induced by a broad spectrum of insults has been clearly demonstrated by the findings demonstrating the increased sensitivity of Nrf2\(^{-/-}\) mice to a variety of insults (14). Recently, the essential role of Nrf2 in protecting against diabetic vascular diseases has emerged. Activation of Nrf2 by sulforaphane suppressed hyperglycemia-induced ROS and metabolic dysfunction in human microvascular endothelial cells (16). Using primary cardiomyocytes isolated from Nrf2\(^{+/+}\) and Nrf2\(^{-/-}\) mice, Ma and coworkers demonstrated that Nrf2 conferred protection against high glucose-induced oxidative damage (17). In another study, Yoh et al reported a beneficial role of Nrf2 against diabetes using a STZ-induced diabetes model (18). In their study, higher urinary nitric oxide metabolites, higher levels of ROS, and greater degree of nitrosative DNA damage were detected in STZ-treated Nrf2\(^{-/-}\) mice, than in STZ-treated Nrf2\(^{+/+}\) mice (18).

During the later stages of DN, TGF-\(\beta\)1 overexpression, ECM deposition, and loss of glomerular architecture define glomerulosclerosis (10). Mounting evidence suggests a role of TGF-\(\beta\)1 in the progression of DN and glomerulosclerosis by controlling production of many ECM proteins (19-24). For instance, an anti-TGF-\(\beta\)1 antibody were reported to be useful in inhibiting glomerulonephritis by blocking ECM production in a mesangial proliferative glomerulonephritis model (25). Interestingly, a recent report indicated the interplay between the TGF-\(\beta\)1 and Nrf2 pathways. Activation of
the TGF-β1 pathway was reported to inhibit Nrf2-dependent expression of γ-glutamylcysteine synthetase catalytic subunit (GCLC), resulting in elevated ROS levels. This in turn, further activated the TGF-β1 pathway, leading to excessive production of ECM (26). On the other hand, activation of Nrf2 is able to inhibit the function of TGF-β1 in a liver fibrosis mouse model (27). Thus, it is conceivable that activation of the Nrf2-mediated antioxidant response is beneficial in preventing or slowing down the progression of DN by reducing ROS and TGF-β1-mediated ECM production. In this report, we show that kidneys from DN patients are under oxidative stress. Consistent with the notion that Nrf2 is beneficial in suppressing the progression of DN, we observed that Nrf2−/− mice suffered greater STZ-induced renal damage. Furthermore, a negative role of Nrf2 in controlling the promoter activity of TGF-β1 and ECM production was demonstrated in HRMCs.

**Research Design and Methods**

**Patients and Renal Histology:** The DN kidney tissues were obtained from 8 patients with proteinuria that underwent a renal biopsy for diagnosis of DN, at the Department of Pathology, Shanghai Medical College, Fudan University in 2007 or 2008. All cases were diagnosed by 2 individual pathologists in a double-blind manner. Eight patients who underwent kidney transplants were biopsied one year later to insure normal function of their transplanted kidney. These biopsies were then used as the normal kidney specimens in our study. Permission to use the fixed tissue sections for research purposes was obtained and approved by the Ethics Committee from Shanghai Medical College, Fudan University, China, and a written consent form was obtained from all patients. The paraffin sections were stained with hematoxylin and eosin (HE), congo red, periodic acid Schiff (PAS), and immunofluorescence depositions for diagnosis of DN.

**Animals and treatments:** Nrf2−/− mice in C57BL/6 background were gifts from Dr. Jeff Chan (University of California, Irvine) (28). Nrf2+/+ and Nrf2−/− mice (8 mice per group), at 8 weeks of age, were intraperitoneally injected with STZ (50 mg/kg, pH 4.5) dissolved in sodium citrate or injected with vehicle (sodium citrate only) for 5 consecutive days. At 16 weeks post-injection, the mice were euthanized and the kidneys were isolated.

**Blood glucose and urine albumin-to-creatinine ratio (UACR) measurement:** Blood glucose levels from the tail-vein were measured by the OneTouch Blood Glucose Monitoring System (LifeScan Inc., Milpitas, CA) at 3, 5, 8, 12 and 16 weeks post-injection. The mice fasted for 4 h prior to blood glucose measurement. Freshly voided spot urine samples were collected at 1, 8 and 16 weeks post-injection. Urine albumin and creatinine levels were measured by ELISA kits (albumin: Bethyl Laboratories Inc., Houston, TX; creatinine: Exocell, Philadelphia, PA). The UACR was expressed as the ratio of albumin to creatinine.

**Mouse renal histology and detection of oxidative DNA damage:** Both kidneys from each mouse were isolated and cut into 2 halves. The tissue was cut into 4 µm sections and stained with HE, PAS, and Masson’s trichrome. For PAS-stained tissue sections, a five-grade method was used to evaluate the sclerosis in glomeruli as described (29). The
protocol for detection of 8-Oxo-dG requires an additional step: the deparaffinized sections were incubated with proteinase K (10 µg/ml) for 30 min at 37 °C and RNase A (100 µg/ml) for 1 h at 37 °C, then exposed to 2N HCl for 5 min at RT, followed by the immunohistochemical analysis (IHC). Urinary 8-Oxo-dG is measured using LC-MS/MS.

IHC and antibodies: Kidney tissues were fixed in 4% buffered paraformaldehyde and embedded in paraffin. The deparaffinized sections were boiled in sodium citrate buffer. The primary antibody was used in a dilution of 1:100 for 1 h at 37 °C and 4 °C overnight. For human tissues, an anti-Nrf2 antibody from Abcam (Cambridge, MA) was used. For mouse tissues, an anti-Nrf2 polyclonal antibody was used (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Antibodies against 8-Oxo-7, 8-dihydro-2'-deoxyguanosine (8-Oxo-dG) (Gaithersburg, MD) and FN (Calbiochem, San Diego, CA) were purchased from commercial sources. NQO1, aldose reductase (AR), γGCS and GAPDH antibodies were all purchased from Santa Cruz.

qRT-PCR and immunoblot assay: Total RNA from kidney tissues or HRMCs was extracted using Trizol solution. Equal amounts of RNA (2 µg) was reverse-transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, IN). Taqman probes and primers used in this study are listed in Table 1. For the bar graph in Fig 5A, the data represent the average of 8 mice in the same group, each with duplicated samples for qRT-PCR. The data were expressed as relative mRNA levels normalized to β-actin, and the value from the Nrf2+/+ control was set as 1. For the in vitro study with HRMCs, the experiments were repeated three times with duplicate samples. For immunoblot analysis the frozen kidneys were homogenized in lysis butter (0.1 M Tris buffer (pH7.4), 0.1 mM EDTA) in the presence of 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Roche, IN). Protein concentration was determined using the Bio-Rad DC protein Assay (Bio-Rad, CA). 300 µg of protein was loaded into each well and subjected to immunoblot analysis. Each lane in Fig 5C contained proteins extracted from the kidney of individual mice.

Cell culture, ROS detection, siRNA transfection, and luciferase reporter gene assay: HRMCs were purchased from ScienCell Research Laboratories (Carlsbad, CA) and maintained in RPMI 1640 supplemented with 2% FBS and mesangial cell growth supplement (MCGS) (ScienCell Research Laboratories). After growth-arrested in 0.5% FBS for 24 h, cells were cultured in either low (1 g/l) or high (5.4 g/l) glucose DMEM medium for an additional 24 h before harvest. For ROS measurement, cells were then incubated for 30 min with dichlorofluorescein (DCF 5 µg/ml) (Sigma, MO) and the fluorescence intensity was measured by flow cytometry. Nrf2 siRNA and HiPerfect transfection reagent were purchased from Qiagen (Valencia, CA) and transfection of Nrf2 siRNA was performed according to the manufacture’s instruction. For luciferase reporter gene assay, HRMCs were transfected with plasmids for TGF-β1-promoter-firefly luciferase and renilla luciferase, along with different amounts of an expression vector for Nrf2, then firefly and renilla luciferase activities were measured using a dual-luciferase reporter gene assay system.
Statistical test: Results were expressed as mean ± SD. Statistical tests were performed using SPSS 10.0. Unpaired student t-tests were used to compare the means of two groups. One-way ANOVA was applied to compare the means of three or more groups. The Wilcoxon (Gehan) statistical test was used to analyze survival rate. *P< 0.05 was considered to be significant.

RESULTS

The glomeruli of human DN patients were under oxidative stress and underwent significant pathological changes: In total, tissues from 8 normal and 8 DN kidneys were selected for this study. The selected clinical characteristics of patients are shown in Table 2. Pathological and IHC were performed (Fig 1). Compared to normal glomeruli, the glomeruli from DN patients showed significant morphological changes (Fig 1, compare panel a to b). Two massive K-W nodules were formed inside the glomerulus of DN patients (Fig 1b, white arrows). It has been reported that overproduction of ROS is one of the major factors mediating tissue damage in diabetes mellitus (30). Thus, Nrf2 is likely activated in the glomeruli of DN patients. Next, Nrf2 expression in normal and DN glomeruli was measured using IHC. Nrf2 was hardly expressed in normal glomeruli, whereas it was upregulated in DN glomeruli (Fig 1, compare panel c to d). In addition, cells with high expression of Nrf2 in the nucleus were identified as mesangial cells (Fig 1, panel d, arrows). NQO1, a well-studied Nrf2 target gene, was also activated in glomeruli of DN patients (Fig 1, compare panel e to f), indicating the activation of the Nrf2-mediated antioxidant response. Next, oxidative DNA damage was measured using IHC with an anti-8-Oxo-dG antibody. Nuclear staining was detected in some cells of the glomeruli from DN patients, indicating the DN kidney is under oxidative damage (Fig 1, panel g to h). However, the number of nuclear stained cells was low in DN patients because of the massive glycogen deposition inside the glomeruli (Fig 1, panel h). Together, these results demonstrated that the glomeruli of DN patients are under severe oxidative stress and the Nrf2-mediated antioxidant response is activated.

Nrf2−/− mice suffered greater renal damage induced by STZ, compared to Nrf2+/+ mice: During the course of 16 weeks, some mice in both STZ-treated Nrf2+/+ and Nrf2−/− groups died, but the survival rate between the two groups did not show any statistical difference (Fig 2A, *P=0.9477). Blood glucose levels, monitored at 3, 5, 8, 12 and 16 weeks post-injection, were significantly increased in both Nrf2+/+ and Nrf2−/− mice following STZ treatment, although no difference in glucose levels was observed between the two genotype groups (Fig 2B, *P<0.05).

At 16 weeks post-injection, mice in the STZ-treated groups had a decrease in their body weight, while the untreated groups gained weight (Fig 2C, *P<0.05). Interestingly, Nrf2−/− mice lost more body weight than Nrf2+/+ mice when treated with STZ (Fig 2C, *P<0.05). Next, the ratio of kidney to body weight, which indicates the enlargement of the kidney, was calculated. The ratio was significantly increased in the STZ-treated groups (Fig 2D, *P<0.05). However, there was no difference in the ratio of kidney to body weight between the two
genotypes even in the treated groups, which may be due to the greater decrease in body weight of the Nrf2+/− mice (Fig 2D). As an index of renal function, UACR was measured at 0, 8, and 16 weeks following STZ injection. STZ markedly enhanced UACR at both 8 and 16 weeks post-injection (Fig. 2E *P<0.05). Although no difference was observed between Nrf2+/− and Nrf2+/+ mice at 8 weeks post-injection, the Nrf2−/− mice showed a significantly higher UACR than Nrf2+/+ mice at 16 weeks post-injection (Fig 2E, #P <0.05).

All these results indicate that Nrf2−/− mice suffered greater renal damage, implicating the essential role of Nrf2 in protecting against STZ-induced DN.

Higher levels of oxidative stress and oxidative damage occurred in the glomeruli of Nrf2−/− mice than in Nrf2+/+ mice in response to STZ: Next, STZ-induced oxidative stress were measured by Nrf2 activation and oxidative DNA damage. Nrf2−/− mice did not have any detectable levels of Nrf2 in their glomeruli, confirming the complete deletion of Nrf2 (Fig 3A, panel c and d). Nrf2 expression was greatly enhanced in the glomeruli of the STZ-treated Nrf2+/+ group and Nrf2 nuclear staining was observed (Fig 3A, compare panel a to b, and the insert of panel b). Activation of Nrf2 was confirmed by upregulation of NQO1 in response to STZ treatment in Nrf2+/+ mice, but not in Nrf2−/− mice (Fig3A, panels i-l). To test the role of Nrf2 in ameliorating oxidative damage under the DN condition, oxidative DNA damage was compared in the glomeruli of Nrf2+/+ and Nrf2−/− mice using IHC with an anti-8-Oxo-dG antibody. The results reveal that the STZ-treated Nrf2−/− mice had a greater degree of oxidative damage than the STZ-treated Nrf2+/+ mice (Fig 3A, compare panel f to h). Intriguingly, Nrf2−/− mice displayed higher levels of oxidative damage even in the untreated condition (Fig 3A, compare panel e to g) indicating that the basal level of Nrf2 is essential in protecting against DNA damage induced by intrinsic sources of ROS. Consistent with these results, higher levels of basal and STZ-induced oxidative DNA damage in Nrf2−/− mice were confirmed by measuring the urinary level of 8-Oxo-dG (Fig 3B). These results clearly demonstrate that Nrf2−/− mice had higher production of ROS in response to STZ challenge and were more vulnerable to ROS-induced damage due to loss of Nrf2.

**Nrf2−/− mice had more severe glomerular injury than Nrf2+/+ mice:** Kidneys were isolated and processed for pathological analysis using HE, PAS and Masson’s trichrome staining. Glomerular lesions were detected in both Nrf2+/+ and Nrf2−/− mice following STZ injection in HE-stained tissue sections (Fig 4A, compare panel a to b, and c to d, K-W nodules are labeled with arrows in panels b and d). Next, glycogen deposition was measured by PAS staining (Fig 4A, panels e-h). The severity of glomerulosclerosis was scored using a semiquantitative method and the result is shown in Fig 4B. STZ significantly induced glomerulosclerosis in both genotype groups (Fig 4A compare panel e to f, g to h, Fig 4B, *P<0.05). Moreover, Nrf2−/− mice showed a higher score than Nrf2+/+ mice in response to STZ treatment (Fig 4A, compare panel f to h, Fig 4B, #P<0.05). In addition, K-W nodules were also observed in PAS stained tissues in the STZ-treated groups (Fig 4A, compare panel e to f, and g to h, black arrows in panel f and h). ECM deposition in glomeruli is a hallmark of many renal diseases including...
Therefore, collagens and FN, the major components of ECM, were measured using Masson’s trichrome staining method and IHC with an anti-FN antibody, respectively. Collagen deposition was observed inside the glomeruli of the STZ-treated Nrf2−/− mice, but not in STZ-treated Nrf2+/+ mice (Fig 4A, compare j to l). Slight collagen deposition in the untreated Nrf2−/− mice was also observed (Fig 4A, panel k). In response to STZ treatment, expression of FN was increased in both genotype groups (Fig 3A, compare panel m to n, o to p). Consistent with the observed oxidative damage in untreated Nrf2−/− mice (Fig 3A, panel g), the basal level of FN expression in Nrf2−/− was also higher than that in Nrf2+/+ mice (Fig 4A, compare panel m to o). Collectively, these results demonstrate that Nrf2 is essential in protecting against both basal and STZ-induced glomerular injury.

**Nrf2−/− mice had higher TGF-β1 transcription and FN expression:** Next, the molecular mechanism by which Nrf2 protects against STZ-induced glomerular injury was explored. First, activation of the Nrf2 pathway by STZ-induced ROS was tested. Data shown in Fig 5A represent the average reading of 8 mice in each group. As expected, there was no Nrf2 mRNA detected in Nrf2−/− mice (Fig 5A, Nrf2 panel #p<0.05). In addition, STZ treatment did not induce Nrf2 mRNA expression in Nrf2+/+ mice (Fig 5A, Nrf2 panel #p<0.05). In contrast, NQO1 and GST, downstream genes of Nrf2, were transcriptionally activated in response to STZ in Nrf2+/+ mice, and only slightly in Nrf2−/− mice (Fig 5, NQO1 and GST panels, *p<0.05), indicating the activation of the Nrf2-mediated antioxidant response. Intriguingly, although both basal and STZ-induced levels of NQO1 in Nrf2−/− mice were lower than that in Nrf2+/+ mice, the basal level of GST was similar between these two genotype groups (Fig 5A, NQO1 panel, #p<0.05, and GST panel).

Next, mRNA expression of TGF-β1, FN, and collagen IV were measured. As shown in Fig 5B, the basal level of TGF-β1 in Nrf2−/− mice is higher than Nrf2+/+ mice (Fig 5B, TGF-β1 panel, #p<0.05). STZ treatment induced transcription of TGF-β1 in both Nrf2+/+ and Nrf2−/− mice, and the highest transcription was observed in the STZ-treated Nrf2−/− mice (Fig 5B, TGF-β1 panel, *p<0.05, #p<0.05). In agreement with the notion that TGF-β1 positively regulates expression of FN, the mRNA expression pattern of FN is similar to that of TGF-β1 (Fig 5B, FN panel, *p<0.05, #p<0.05), demonstrating that FN was overexpressed, especially in the STZ-treated Nrf2−/− mice. To our surprise, although the basal level of collagen IV mRNA expression in Nrf2−/− mice was higher, compared to Nrf2+/+ mice (Fig 5B, collagen IV panel, #p<0.05), there was no difference in collagen IV mRNA expression between the control and STZ-treated groups. It is likely that the observed collagen deposition in the glomeruli of the STZ-treated Nrf2−/− mice in the Masson’s trichrome stained tissues (Fig 4, panel l) came from other types of collagen, rather than collagen IV. In another set of experiments, NQO1 and FN were chosen as representative Nrf2 and TGF-β1 downstream genes, respectively, and their protein levels were measured by immunoblot analysis. NQO1 was induced by STZ treatment in Nrf2+/+ mice while there was no detectable
level of NQO1 in Nrf2+/− mice (Fig 5C, NQO1 panel). Quantification data showed nearly 4 fold induction of NQO1 in response to STZ in Nrf2+/− mice (Fig 5C, lower left panel *P<0.05). The protein level of FN was induced more than 3-4 fold by STZ treatment, both in Nrf2+/+ and Nrf2+/− mice (Fig 5C, FN panel, and lower right panel *P<0.05).

Furthermore, Nrf2−/− mice had both higher basal and STZ-induced levels of FN than Nrf2+/+ mice, with the highest FN expression detected in the STZ-treated Nrf2+/− mice (Fig 5C, FN panel and lower right panel, #P<0.05).

Taken together, these data demonstrate that STZ is able to activate the Nrf2-mediated antioxidant response, which in turn, negatively regulates TGF-β1-mediated ECM production, especially FN.

**Nrf2 was activated by high glucose-induced ROS production in HRMCs.** To further confirm that the activation of Nrf2 by STZ in vivo is due to high glucose-induced ROS production, in vitro experiments were carried out. Since mesangial cells play a crucial role in the initiation and progression of many renal diseases, including DN (31; 33), HRMCs were used for this in vitro study. An enhanced nuclear protein level of Nrf2 was detected in cells growing under high glucose medium compared to low glucose medium (Fig 6A, *P<0.05). Consistent with this result, high glucose induced the mRNA level of NQO1, HO-1 and GST, without affecting Nrf2 mRNA levels (Fig 6B, *P<0.05), indicating the activation of the Nrf2 pathway. This in vitro study recapitulates the observed Nrf2 activation in STZ-treated Nrf2+/− mice and in human renal tissues from DN patient. It is conceivable that high glucose induced Nrf2 activity through ROS production. Thus, ROS levels were measured. Indeed, the cells growing in high glucose medium had substantially higher levels of ROS (Fig 6C, *P<0.05). To further confirm the notion that Nrf2 activation by high glucose is through generation of ROS, N-acetylcysteine (NAC), a ROS scavenger, was included in the medium. As shown in Fig 6D, NAC inhibited the activation of high glucose-induced Nrf2 and NQO1 (Fig 6D, *P<0.05, #P<0.05). Collectively, these results indicate that hyperglycemia is able to activate the Nrf2 pathway through generation of ROS.

**Nrf2 negatively regulated TGF-β1 and FN in HRMCs:** To confirm the negative effects of Nrf2 on TGF-β1, as observed in the in vivo study shown in Fig 5B, regulation of the TGF-β1 promoter activity by Nrf2 was studied using luciferase reporter gene analysis. Overexpression of Nrf2 inhibited the promoter activity of TGF-β1 in a dose-dependent manner (Fig 7A, upper panel, *P<0.05). Overexpression of Nrf2 was confirmed by immunoblot analysis with an anti-HA antibody (Fig 7A, lower anti-HA panel). Consistent with the result obtained from overexpressed Nrf2, induction of endogenous Nrf2 by tert-butylhydroquinone (tBHQ) inhibited the promoter activity of TGF-β1 in a dose-dependent manner (Fig 7B, upper panel, *P<0.05, and lower anti-Nrf2 panel). Consistent with the Nrf2-mediated negative regulation of the TGF-β1 promoter, Nrf2 also negatively regulated the protein level of FN, a TGF-β1-downstream gene (Figs 7A and 7B, FN panel). In addition, changes in TGF-β1 mRNA in response to reduced expression of Nrf2 by Nrf2-siRNA were measured in cells growing in low and high glucose medium. As expected, cells growing in high glucose medium had a higher level of
TGF-β1 expression (Fig 7C, *P<0.05). Knockdown of Nrf2 significantly enhanced the mRNA level of TGF-β1 in both conditions (Fig 7C, #P<0.05), again demonstrating the negative effect of Nrf2 on TGF-β1 expression. Next, another parallel set of samples was used for immunoblot analysis. High glucose induced Nrf2, AR, γGCS, and NQO1, as well as FN (Fig 7D, compare lane 1 to 3, *P<0.05). Nrf2-siRNA reduced the protein levels of Nrf2, AR, γGCS, NQO1, while it enhanced FN in both low and high glucose medium (Fig 7D, compare lane 1 to 2, lane 3 to 4, #P<0.05).

**DISCUSSION**

In the present study, a protective role of Nrf2 against DN is clearly demonstrated through multiple approaches. First, human kidney tissues were utilized and the results indicate that the glomeruli of human DN patients were under oxidative stress, as demonstrated by oxidative DNA damage and activation of the Nrf2-mediated pathway. Second, a STZ-induced diabetes mouse model in Nrf2⁻/⁻ mice was used to demonstrate the importance of Nrf2 in alleviating hyperglycemia-induced renal damage. Nrf2⁻/⁻ mice had higher ROS production and suffered from greater oxidative DNA damage. This was recapitulated in HRMCs using media containing high glucose, showing an increase in ROS, which was attenuated when the cells were treated with the antioxidant supplement, NAC (Fig 6D). In addition, Nrf2⁻/⁻ mice manifested severe proteinuria and glomerulosclerosis, and thus, suffered a greater degree of renal injury, compared to Nrf2⁺/+ mice. Consistent with our findings that Nrf2 plays an important role in alleviating renal damage caused by ROS production, Liu et al. demonstrated that renal function, histology, vascular permeability, and survival were significantly worse in Nrf2⁻/⁻ mice under ischemic conditions, which was blocked by NAC or glutathione treatment (34). To further verify that Nrf2 protects against ROS, a future in vivo study should be conducted using NAC or glutathione to confirm that antioxidants reduce ROS-induced damage in Nrf2⁻/⁻ mice.

Finally, the possibility that Nrf2 negatively regulates TGF-β1 and its downstream events, such as ECM production, was explored both in mice and in cultured mesangial cells. Strikingly, the basal mRNA level of TGF-β1, FN and collagen IV in Nrf2⁻/⁻ mice was increased significantly compared to Nrf2⁺/+ mice, indicating the negative effect of Nrf2 on the TGF-β1 pathway. This notion was further confirmed by the higher basal and induced protein levels of FN in Nrf2⁻/⁻ mice. In HRMC, high glucose induced ROS production, and activated expression of Nrf2 and its downstream genes. Furthermore, the activation or overexpression of Nrf2 inhibited the promoter activity of TGF-β1 in a dose-dependent manner, whereas knockdown of Nrf2 by siRNA enhanced TGF-β1 transcription and FN production. This work clearly establishes a critical role of Nrf2 in protecting against DN.

Since Nrf2 and NQO1 were upregulated exclusively in the kidneys of all 8 DN patients, it is most likely that the Nrf2-mediated antioxidant response pathway is intact in DN patients. Then the question is why the Nrf2-mediated antioxidant response failed to protect these DN patients? We believe, that activation of Nrf2 before disease development or during the early stage of DN is the key for intervention to prevent ROS-induced damage.
and DN progression. At the late stage of DN, the Nrf2-mediated protective mechanism is saturated by excessive ROS, resulting in renal damage. Elucidation of the Nrf2-mediated defense system as a protective mechanism against DN will help us to develop novel therapeutic interventions targeting Nrf2 to prevent or slow the progression of DN.

Based on the findings from this study, we propose a model by which Nrf2 confers protection again DN. Under hyperglycemic conditions, glomerular cells produce excessive amounts of ROS, which elicits many responses having both beneficial and harmful outcomes in terms of renal function. Upregulation of TGF-β1 by ROS will lead to excessive production of ECM, resulting in glomerular sclerosis/fibrosis, and ultimately loss of renal function. In addition, ROS may directly damage macromolecules, such as DNA, proteins, and lipids, which results in renal cell death and loss of function. To prevent ROS-induced damage, cells have evolved the Nrf2-mediated defense mechanism to cope with deleterious conditions. Activation of Nrf2 by stress improves renal function through at lease the following two mechanisms: one is to neutralize ROS and thus, reduce ROS-mediated damage, and another way is to down-regulate TGF-β1 and therefore, alleviate ECM production. Currently, the detailed mechanism by which Nrf2 down-regulates the transcription of TGF-β1 is unclear. Whether Nrf2 negatively regulates TGF-β1 by direct binding to the promoter region of TGF-β1, or by reducing ROS and ROS-induced upregulation of TGF-β1, are currently under investigation.

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FIGURE LEGENDS

Fig 1. Significant pathological changes and activation of Nrf2 pathway in the glomeruli of human DN patients

Renal biopsy samples were fixed and cut into 2 µm sections, the sections were subjected to HE staining (panels a and b) and IHC with anti-Nrf2 (Panels c and d), anti-NQO1 (panels e and f) and anti-8-Oxo-dG (panels g and h) antibodies. The images shown are representatives of 8 normal kidney tissues patients (panels a, c, e and g) and 8 kidney tissues from DN patients (panels b, d, f and h). Magnification is 400×. White and black arrows (panel b and d) indicate K-W nodules and the Nrf2 positive nuclei, respectively.

Fig 2. Nrf2−/− mice suffered greater renal damage by STZ, compared to Nrf2+/+ mice

During the course of 16 weeks following STZ injection, the survival rate of mice was recorded and plotted (Fig 2A). Tail-vein blood glucose levels were monitored at 3, 5, 8, 12, and 16 weeks post-injection (Fig 2B). Mice were sacrificed at 16 weeks post-injection. Whole body and kidney weight were measured. The body weight in four different groups at 16 weeks post-injection was shown in Fig 2C. The ratio of kidney to body weight was calculated (Fig 2D). UACR was also measured at 0, 8, and 16 weeks post-injection (Fig 2E). *P<0.05 untreated vs. STZ-treated; #P<0.05 Nrf2+/+ vs. Nrf2−/−. Data are expressed as mean ± SD (N=8).

Fig 3. Higher levels of oxidative stress and oxidative damage occurred in the glomeruli of Nrf2−/− mice than in Nrf2+/+ mice in response to STZ

At 16 weeks post-injection, Nrf2+/+ and Nrf2−/− mice were sacrificed and kidneys were isolated. Kidney tissue sections from each mouse were used for IHC with anti-Nrf2 (Fig 3A, panels a-d), anti-8-Oxo-dG (Fig 3A, panels e-h) and anti-NQO1 (Fig 3A, panels i-l) antibodies. Nuclear staining of Nrf2 is shown in the insert (Fig 3A, panel b). Each image is a representative of 8 kidney tissue sections from 8 mice in each group. Urinary 8-Oxo-dG was detected by LC-MS/MS (Fig 3b).

Fig 4. Nrf2−/− mice had more severe glomerular injury than Nrf2+/- mice

Kidney tissue sections were subject to HE (Fig 4A, panels a-d), PAS (Fig 4A, panels e-h), and trichrome staining (Fig 4A, panels i-l), as well as IHC with an anti-FN antibody (Fig 4A, panels m-p). Each image is a representative of 8 kidney tissue sections from 8 mice in each group. PAS-stained tissues were used for semiquantitative scoring as described in the Materials and Methods section. Glomerulosclerosis index for four different groups is shown in Fig 4B. In total, 30 glomeruli were scored for each mouse. *P<0.05 untreated vs. STZ-treated; #P< 0.05 Nrf2+/- vs. Nrf2−/−. Data are expressed as mean ± SD (N=8).

Fig 5. Nrf2−/− mice had higher TGF-β1 transcription and FN expression

The mRNA level of Nrf2, NQO1, and GST (Fig 5A), TGF-β1, FN and collagen IV (Fig 5B) was measured by qRT-PCR. The data presented are relative mRNA level normalized to β-actin mRNA level, and the value from the untreated Nrf2+/- group was set as 1. *P<0.05 untreated vs.
STZ-treated; \(^*P<0.05\) Nrf2\(^{+/+}\) vs. Nrf2\(^{-/-}\). Data are expressed as mean ± SD (N=8). The protein level of FN, NQO1, and GAPDH was measured by immunoblot analysis (Fig 5C, upper panel). Each lane contained total proteins from the kidney of different individual mice. The band intensity was calculated and normalized to GAPDH (Fig 5C, lower panel). The value from the untreated Nrf2\(^{+/+}\) group was set as 1. \(^*P<0.05\) untreated vs. STZ-treated; \(^#P< 0.05\) Nrf2\(^{+/+}\) vs. Nrf2\(^{-/-}\). Data are expressed as mean ± SD (N=3).

**Fig 6. Nrf2 was activated by high glucose-induced ROS production in HRMCs**

Prior to exposure to low (1 g/L) or high glucose (5.4 g/L) DMEM medium, cells were starved for 24 h with low glucose DMEM medium containing 0.5% FBS. Cells were then incubated in low and high glucose DMEM for an additional 24 h. Nuclear and Cytosolic fractions were extracted and subjected to immunoblot analysis using anti-Nrf2, anti-lamin A, and anti-tubulin antibodies (Fig 6A, left panel). The intensity of the bands was calculated and quantified (Fig 6A, right panel). Total mRNA was extracted and used for qRT-PCR for measurement of the mRNA level of Nrf2, HO-1, NQO1, or GST (Fig 6B). ROS level was also measured in these cells growing in low or high glucose medium by DCF/flow cytometry analysis described in the Materials and Methods section (Fig 6C). HRMCs were incubated with N-acetylcysteine (NAC, 50 mM) for 24 h. Total cell lysates were subjected to immunoblot analysis using anti-Nrf2, anti-NQO1 and anti-GAPDH antibodies (Fig 6D, left panel). The intensity of the bands was quantified (Fig 6D, right panel).

**Fig 7. Nrf2 negatively regulated TGF-β1 and FN in HRMCs**

HRMC cells growing were transfected with plasmids for TGF-β1-promoter-firefly luciferase and renilla luciferase (internal control), along with different amounts of an expression vector for Nrf2. At 48 h-post transfection, both firefly and renilla luciferase activities were measured (Fig 7A, upper panel). An aliquot of cell lysates were used for immunoblot analysis (Fig 7A, lower panel). HRMCs were transfected with plasmids for TGF-β1-promoter-firefly luciferase and renilla luciferase. Cells were then treated with tBHQ for 16 h prior to the measurement of luciferase at 48 h post-transfection (Fig 7B, upper panel). An aliquot of cell lysates were used for immunoblot analysis (Fig 7B, lower panel). HRMCs were transfected with control- or Nrf2-siRNA. 24 h later, cells were starved for 24 h before incubation with low or high glucose medium for an additional 24 h. Total mRNAs were extracted and qRT-PCR was performed to measure the mRNA level of TGF-β1 (Fig 7C). Another parallel set of cells was collected in lysis buffer for immunoblot analysis with antibodies against Nrf2, AR, γGCS, NQO1, FN, and GAPDH (Fig 7D, upper panel). The intensity of the bands was calculated and quantified (Fig 7D, lower panel). All the experiments were repeated three times and data represent mean ± SD.
Table 1 Primers and probes used for qRT-PCR

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<th>Probe</th>
<th>Primer nucleotide sequences</th>
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Table 2: Selected clinical characteristics of patients in the study group.

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<td>N</td>
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<tr>
<td>Sex (male/female)</td>
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<tr>
<td>Age</td>
<td>59±8</td>
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<tr>
<td>Duration of diabetes</td>
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<tr>
<td>A1C (%)</td>
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<td>SBP (mmHg)</td>
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<td>DBP (mmHg)</td>
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<tr>
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<td>AER (µg/min)</td>
<td>NA</td>
<td>664.2(233.0-769.1)</td>
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</table>

N: no diabetic nephropathy
NA: not available
Fig 2

A

Survival

Nrf2 +/-  
Nrf2 -/-

Time (weeks)

B

Blood glucose (mg/dl)

- Nrf2 +/- control
- Nrf2 +/- STZ
- Nrf2 -/- control
- Nrf2 -/- STZ

0 3 5 8 12 16 weeks

C

Average body weight

0 16 (weeks)

D

Kidney/body weight

control  STZ

E

UCR (ug Albumin/mg Creatinine)

0 8 16 (weeks)

- Nrf2 +/- control
- Nrf2 -/- control
- Nrf2 +/- STZ
- Nrf2 -/- STZ
Fig 7

A

Relative luciferase activity

HA-Nrf2 0 1 2 5 10 ng

B

Relative luciferase activity

tBHQ 0 10 20 30 40 uM

C

Relative TGFβ1 mRNA level

low glucose high glucose

D

HRMC

low glucose high glucose

control siRNA control siRNA

Nrf2 AR γGCS NQO1 FN

GAPDH

1 2 3 4

Relative gene expression

control cSOD-αvβ5 Nrf2 siRNA Nrf2 siRNA

Nrf2 AR γGCS NQO1 FN

GAPDH

Nrf2 AR γGCS NQO1 FN