

Original Articles

Keap1 inhibition attenuates glomerulosclerosis

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ABSTRACT

Background. NFE2-related factor 2 (Nrf2) is a master regulatory transcription factor for antioxidant genes. Inhibition of its adaptor protein, Kelch-like ECH-associated protein 1 (Keap1), activates Nrf2. Podocyte injury triggers the progressive deterioration of glomerular damage toward glomerulosclerosis. We examined whether modulation of the Keap1-Nrf2 system has an impact on this process.

Methods. *Nrf2* null-mutant (KO) and *Keap1* hypomorphic knockdown (KD) mice were crossed with NEP25 mice, in which podocyte-specific injury can be induced by an immunotoxin.

Results. Thiobarbituric acid reactive substances, 8-hydroxydeoxyguanosine and phosphorylated JNK were increased in the injured NEP25 kidney. Real-time PCR revealed that *Keap1* KD upregulated Nrf2 target genes, including *Gclc*, *Gclm*, *Gstp1*, *Gstp2* and *Nqo1* in the glomerulus. However, podocyte injury did not upregulate these genes in *Keap1* wild-type mice, nor did it further increase the expression of those genes in *Keap1* KD mice. Three weeks after the induction of podocyte injury, glomerulosclerosis was considerably more attenuated in *Keap1* KD mice than in control mice (median sclerosis index, 0.27 versus 3.03, on a 0–4 scale). *Keap1* KD mice also showed considerably preserved nephrin staining (median index, 6.76 versus 0.91, on a 0–8 scale) and decreased glomeruli containing desmin-positive injured podocytes (median

percentage, 24.5% versus 85.8%), along with a decrease in mRNAs for *Fn1*, *Tgfb1*, *Col4a4* and *Col1a2*.

Conclusions. Thus, podocyte injury cannot effectively activate Nrf2, but Nrf2 activation by *Keap1* knockdown attenuates glomerulosclerosis. These results indicate that the Nrf2-Keap1 system is a promising drug target for the treatment of chronic kidney diseases.

Keywords: antioxidant genes, glomerulosclerosis, hypomorphic allele, oxidative stress

INTRODUCTION

The histology of end-stage renal failure is typically characterized by glomerulosclerosis. Many studies have shown that podocyte injury is a key step triggering the progression of glomerulosclerosis [1–5]. A number of factors, including genetic, mechanical, and immunological stresses such as toxins, as well as oxidative stress, can cause podocyte injury [6–11].

Kelch-like ECH-associated protein 1 (Keap1) is a cytoplasmic protein that serves as a substrate adaptor molecule for ubiquitin E3 ligase to regulate proteasomal degradation [12, 13]. The most relevant substrate of Keap1 is the transcriptional factor NFE2-related factor 2 (Nrf2), which serves as a master regulator of phase II detoxification and antioxidative stress enzymes/proteins [14, 15]. Without oxidative stress,

Keap1 suppresses Nrf2 activity by promoting ubiquitination of Nrf2 and subsequent degradation by the proteasome. Upon exposure to oxidative stress, reactive cysteine residues in Keap1 become modified, which leads to a decline in the E3 ligase activity, stabilization of Nrf2 and the subsequent robust induction of cytoprotective genes. These include the cystine transporter, enzymes for glutathione production and enzymes metabolizing oxidative intermediates, such as NAD(P)H:quinone oxidoreductase 1 (NQO1) [16–18].

Indeed, Nrf2-mediated transcriptional responses have been shown to have protective effects in various experimental diseases, including lipopolysaccharide-induced sepsis (shock) [19, 20], oxidative lung injury and fibrosis, asthma, smoking-induced emphysema [21–25], and brain ischemia-reperfusion injury [26, 27]. These findings are all derived from studies with *Nrf2* knockout mice or pharmacological activation of Nrf2 by small-molecule compounds.

In *Keap1*-null mice, Nrf2 constitutively accumulated in nuclei, and Nrf2 target genes were constitutively activated [15]. However, the knockout mice were found to die in the neonatal stage, mainly due to hyperkeratosis in the upper digestive tract. The phenotypes seen in the knockout mice were all canceled in the double-mutant mice that were null for the *Keap1* and *Nrf2* genes, indicating that *Keap1*-null phenotypes are the result of the constitutive activation of Nrf2 [15]. To generate *Keap1* conditional knockout mice, we previously generated mice carrying a mutant allele (*Keap1^{loxP}*) in which loxP sequences and the *EGFP* gene were inserted into an intron of the *Keap1* gene. Unexpectedly, this allele was found to be hypomorphic. Thus, *Keap1^{loxP/-}* mice, even in the absence of Cre recombinase, displayed a very low level of *Keap1* gene expression, resulting in a constitutive and ubiquitous activation in Nrf2 [28]. As *Keap1^{loxP/-}* mice (designated as *Keap1* knockdown mice) are vital and have no obvious abnormal phenotype, they can provide an opportunity to examine the effect of constitutive Nrf2 activation.

Several papers reported that the Keap1-Nrf2 system is involved in kidney diseases. Thus, *Nrf2* knockout mice are more sensitive to experimental diabetic nephropathy [29], and Nrf2-activating compounds (sulforaphane or cinnamic aldehyde) attenuated the progression of renal damage [30]. Ischemia/reperfusion renal injury was exaggerated in *Nrf2* knockout mice, and the injury was attenuated by treatment with antioxidants (*N*-acetyl-cysteine or glutathione) in these mice [31]. However, few studies have been reported to date about whether the Keap1-Nrf2 system plays a role in podocyte injury and the subsequent development of glomerulosclerosis [32], the common pathogenic pathway to chronic renal failure, especially in nondiabetic and nonischemic conditions.

Previously, we established a mouse model of selective and inducible podocyte injury (NEP25) [33]. NEP25 mice express hCD25 selectively on podocytes. After injection of the hCD25-targeted immunotoxin LMB2, only hCD25-expressing podocytes are selectively injured. With a low dose [0.625 ng/g body weight (BW)] of LMB2, NEP25 mice developed moderate proteinuria, which peaked 1–2 weeks after the injection and gradually decreased. After 3–4 weeks, some glomeruli developed global or segmental sclerosis, whereas other glomeruli

recovered from the podocyte injury and showed a normal structure [33]. The injury induced by this low dose of LMB2 can be attenuated by angiotensin II inhibition [34]. To clarify whether the Keap1-Nrf2 system is involved in the injury and recovery process of podocytes and subsequent development of glomerulosclerosis, we performed histological and morphological analysis with *Keap1^{loxP/-}/Nep25* and *Nrf2^{-/-}/Nep25* mice treated with the low dose of LMB2.

MATERIALS AND METHODS

Animal experiments

Keap1^{loxP/-} and *Nrf2^{-/-}* mice were previously reported [14, 28, 35]. To induce podocyte-specific injury, *Nrf2^{-/-}* or *Keap1^{loxP/-}* mice were mated with NEP25 mice on a C57 BL/6 genetic background [33]. Genotyping for *Nrf2^{-/-}*, *Keap1^{-/-}* and NEP25 was performed on tail DNA by PCR as previously reported [14, 15, 33]. *Keap1^{loxP}* allele was identified by PCR with primers, 5'-GAAGCAGCAGACTTCTTCAAGTC-3' and 5'-TGGCGGATCTTGAAGTTCACCTTG-3'.

Ten mice (5 males and 5 females) carrying *Keap1^{+/+}/Nep25* and 14 mice (6 males and 8 females) carrying *Keap1^{loxP/-}/Nep25* (all 3–5 months of age) were injected with 0.625 ng/g BW of LMB2. Twenty-four-hour urine was collected before and 7, 14 and 21 days after the injection of LMB2. The animals were euthanized 21 days after the injection. Three *Keap1^{loxP/-}/Nep25* mice were excluded from the analysis because they were found to have unilateral hydronephrosis on autopsy, which is frequently observed in *Keap1^{loxP/-}* mice without podocyte injury (unpublished observation). The remaining 11 *Keap1^{loxP/-}/Nep25* mice showed no appreciable phenotype that indicated ureteral obstruction—such as enlarged calyx, thinning of medulla and ectopic staining of Tamm–Horsfall protein on the glomerulus (data not shown)—and were therefore subjected to the following analysis. A similar experiment with the same protocol was duplicated using eight *Keap1^{+/+}/Nep25* mice (all females) and five *Keap1^{loxP/-}/Nep25* mice (two males and three females). In addition, nine *Nrf2^{-/-}/Nep25* mice (four males, five females) and six *Nrf2^{+/+}/Nep25* mice (three males, three females) were injected with 0.625 ng/g BW of LMB2, and similarly analyzed with the same protocol.

Separately, to evaluate the expression level of *Keap1*, *Nrf2* and Nrf2 target genes, three *Keap1^{+/+}/Nep25* and three *Keap1^{loxP/-}/Nep25* mice were injected with 0.625 ng/g BW of LMB2, and 5 days later, glomeruli were isolated by perfusing with Dynabeads. Glomeruli were also isolated from six *Keap1^{+/+}/Nep25* and six *Keap1^{loxP/-}/Nep25* mice without LMB2. RNA was isolated from the glomeruli and subjected to real-time PCR analysis, as described below.

Urinalysis

Concentration of creatinine in urine was determined by enzymatic method in an outside laboratory (SRL, Tokyo, Japan). Since we used both male and female mice in the study

and adult male mice excrete a larger and variable amount of small molecular weight proteins, we measured albumin concentration rather than total protein to evaluate proteinuria in this study. Urinary albumin concentration was measured by the nephelometric method as follows. Urine samples were incubated with a rabbit polyclonal anti-mouse albumin antibody (Cappel, Durham, NC, USA) in PBS at room temperature. The samples were then illuminated with an LED light, and the intensity of the scattered light was measured at 840 nm with a Behring Nephelometer (Siemens Health Care Diagnostics, Norwood, MA, USA). The measurements were highly correlated with the total protein concentration in female samples measured by the pyrogallol red method (Supplementary Figure S1), validating the accuracy of the measurements. This supports the reliability of the nephelometric method when used for albumin measurement.

Statistical analysis

Urinary albumin/creatinine ratios showed a logarithmic normal distribution. For these data, geographic means and 95% confidence intervals are reported in the text. To compare between two groups, MANOVA was used after logarithmic transformation. To compare the sclerosis and nephrin staining indices, pJNK and TBARS between the two groups, nonparametric Mann-Whitney *U*-test was used. For these data, medians, 25 percentiles and 75 percentiles are reported. One-way ANOVA was used to compare the mRNA level among four groups. Values were considered significant at $P < 0.05$. Other methods are described in the Supplementary data.

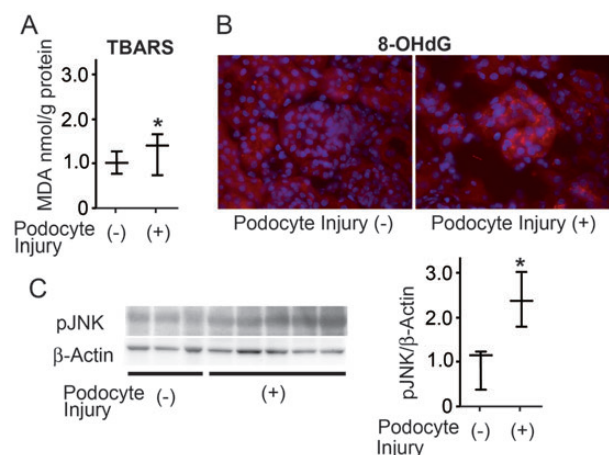


FIGURE 1: Oxidative stress is induced in NEP 25 kidneys after podocyte injury. NEP25 mice were injected with 2.5 ng/g BW of LMB2, and the kidneys were harvested 7 days later. Kidneys from untreated NEP25 mice were used as controls. (A) The levels of TBARS were increased in NEP 25 kidneys after podocyte injury. (B) Immunostaining for 8-OHdG (red) was enhanced in glomeruli and tubules in the NEP25 kidney after podocyte injury but not in the control kidney. (C) Western blot analysis revealed that pJNK was higher in NEP 25 kidneys after podocyte injury. The horizontal bars represent the median and interquartile ranges. * $P < 0.05$ compared with untreated NEP25 mice.

RESULTS

Podocyte injury induced oxidative stress in the kidney in NEP25 mice

We induced podocyte injury by injecting LMB2 into NEP25 mice and then quantified the level of TBARS, which is produced by lipid oxidation [36], in the whole kidney. The level of TBARS was significantly greater (1.6-fold) in the NEP25 kidney after podocyte injury than in the control (Figure 1A). Immunostaining revealed that 8-OHdG, a marker of oxidative DNA damage [37], was enhanced in glomeruli and tubules of NEP25 mice after podocyte injury but not in the control (Figure 1B). In addition, western blot analysis showed that pJNK, which is induced by oxidative stress, was increased by 2.4-fold in the NEP25 kidney after podocyte injury when compared with the control (Figure 1C). These data indicate that oxidative stress is induced in response to podocyte injury in kidneys from NEP25 mice.

Enhancement of Nrf2 signals in the glomeruli of Keap1 knockdown mice

We quantified *Keap1* mRNA by real-time RT-PCR in isolated glomeruli. As expected, *Keap1* mRNA was markedly lower in *Keap1*^{loxP/-}/*Nep25* than in *Keap1*^{+/+}/*Nep25* glomeruli (Figure 2A). We next analyzed nuclear Nrf2 by western blot analysis in the glomeruli and livers of *Keap1*^{loxP/-}/*Nep25*, *Keap1*^{+/+}/*Nep25* and *Nrf2*^{-/-}/*NEP25* mice (Figure 2B). There was clear Nrf2 expression in *Keap1*^{+/+}/*Nep25* liver and even greater expression in *Keap1*^{loxP/-}/*Nep25* liver. Nuclear Nrf2 was not detectable in glomeruli from *Keap1*^{+/+}/*Nep25* mice but was increased in *Keap1*^{loxP/-}/*Nep25* glomeruli. The results

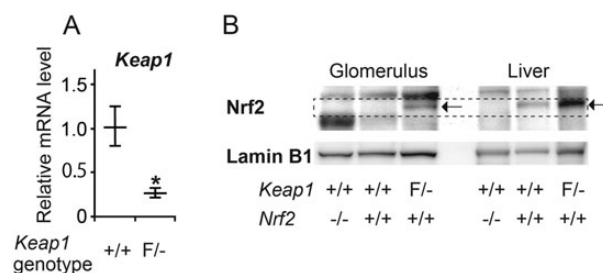


FIGURE 2: Keap1 knockdown activated Nrf2 in the glomerulus. (A) Real-time RT-PCR analysis revealed that the level of glomerular *Keap1* mRNA was remarkably lower in *Keap1*^{loxP/-}/*Nep25* (F/-) than in *Keap1*^{+/+}/*Nep25* (+/+). The horizontal bars represent the average and standard deviations. * $P < 0.05$ compared with *Keap1*^{+/+}. (B) Western blot analysis for nuclear Nrf2 in the glomerulus and the liver. All samples were electrophoresed in the same gel and transferred to the same filter. At the position indicated by the dashed rectangle, a clear band was observed for Nrf2 from livers of *Keap1*^{+/+}/*Nep25* mice, but not from *Nrf2*^{-/-}/*Nep25* mice, whereas from *Keap1*^{loxP/-}/*Nep25* mice, the band was intensified (shown by the arrow). The accompanying larger bands are nonspecific; glomerular nuclei contain larger amounts of the protein causing the nonspecific bands. Nrf2 expression (indicated by the arrow) was only observed in *Keap1*^{loxP/-}/*Nep25* glomeruli. Nrf2 was undetectable in both wild-type and *Nrf2*^{-/-} glomeruli, using this method.

indicate that *Keap1* knockdown activates Nrf2 in the glomeruli of NEP25 mice.

We next quantified nine mRNAs from Nrf2 target genes by real-time RT-PCR in glomeruli. Without LMB2 injection, the expression of *Gclc*, *Gclm*, *Gstp1*, *Gstp2* and *Nqo1* was considerably higher in *Keap1^{loxP/-}/Nep25* than in *Keap1^{+/+}/Nep25* glomeruli, confirming that Nrf2 was indeed activated in *Keap1^{loxP/-}* glomeruli. The increase was, on average, 2.48-fold for *Gclc*, 2.33-fold for *Gclm*, 3.79-fold for *Gstp1*, 3.08-fold for *Gstp2* and 10.37-fold for *Nqo1*, whereas the expression level of *Gpx1*, *Gsta4*, *Hox1* and *Prdx1* was comparable between the two mouse strains (Figure 3).

Podocyte injury induced by LMB2 did not increase the expression of Nrf2 target genes in *Keap1^{+/+}/Nep25* glomeruli. mRNAs for *Gclc*, *Gclm*, *Gstp1*, *Gstp2* and *Nqo1* did not further increase after podocyte injury in *Keap1^{loxP/-}/Nep25* glomeruli, although they are higher than those in *Keap1^{+/+}/Nep25* glomeruli with podocyte injury. These results indicate that podocyte injury caused by LMB2 cannot effectively activate the endogenous Nrf2 in glomeruli.

No significant difference was observed in the levels of glomerular *Nrf2* mRNA expression between *Keap1^{loxP/-}/Nep25* and *Keap1^{+/+}/Nep25* mice at baseline, and LMB2 injection did not change the levels of *Nrf2* mRNA expression in both groups in this period (Supplementary Figure S2).

Effect of *Keap1* knockdown on the progression of glomerulosclerosis

Without LMB2, *Keap1^{loxP/-}/Nep25* mice showed no abnormal phenotype in urinary albumin (Figure 4), renal histology, and nephrin and desmin staining (data not shown).

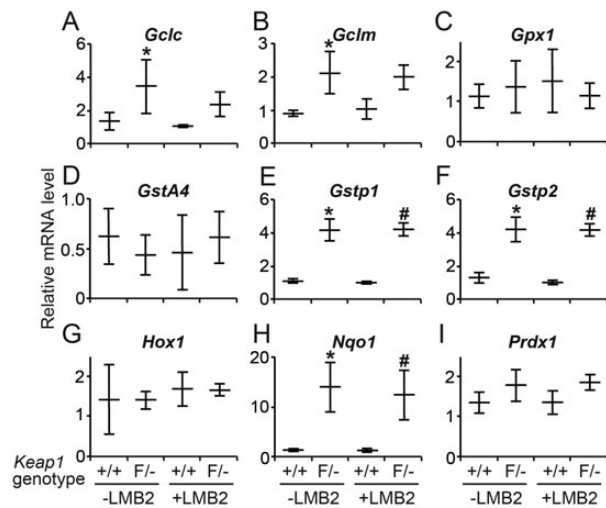


FIGURE 3: Relative mRNA level of Nrf2 target genes in isolated glomeruli. Without LMB2 (-LMB2), *Keap1^{loxP/-}* (F/-) glomeruli showed significantly greater amount of *Gclc* (A), *Gclm* (B), *Gstp1* (E), *Gstp2* (F) and *Nqo1* (G) mRNAs than *Keap1^{+/+}* (+/+) glomeruli. Injection of LMB2 did not change the level of these mRNAs in *Keap1^{+/+}/Nep25* mice, nor did the level further increase in *Keap1^{F/-}/Nep25* mice. The horizontal bars represent the average and standard deviations. *P < 0.05 compared with *Keap1^{+/+}* (-LMB2) and #P < 0.05 compared with *Keap1^{+/+}* (+LMB2).

After injection of 0.625 ng/g BW of LMB2, both *Keap1^{loxP/-}/Nep25* and *Keap1^{+/+}/Nep25* mice showed similarly moderate albuminuria, which peaked 1–2 weeks after the injection. The urinary albumin/creatinine ratio was not different between the two groups at any time points (Figure 4). A recent study reported that bardoxolone methyl, an Nrf2 inducer, reduces tubular albumin reabsorption by suppressing megalin in *Cynomolgus* monkeys [38]. We therefore examined megalin and albumin in a separate set of *Nrf2^{-/-}*, *Keap1^{loxP/-}* and wild-type mice without podocyte injury. Notably, there was no appreciable difference in baseline immunoreactivity for megalin among the three genotypes (Supplementary Figure S3). In addition, the megalin mRNA level was also comparable between *Keap1^{loxP/-}* (relative mRNA level, 2.44 ± 0.46 , $n = 6$) and wild-type mice (2.03 ± 0.78 , $n = 6$). In the *Keap1^{+/+}/Nep25* and *Keap1^{loxP/-}/Nep25* mice 3 weeks after the LMB2 injection, tubular albumin staining was increased reflecting podocyte damage, and megalin staining was decreased in injured tubules as previously reported [39]. These changes similarly occurred in both types of mice, and there was no difference in megalin and albumin staining between the two genotypes (Supplementary Figure S3).

Histological analysis on renal tissue harvested 3 weeks after the LMB2 injection revealed that the degree of glomerulosclerosis was remarkably attenuated in *Keap1^{loxP/-}/Nep25* mice with a median sclerosis index (on a 0–4 scale) of 0.27, compared with *Keap1^{+/+}/Nep25* mice with a median sclerosis index of 3.03 (Figures 5 and 6).

Irreversibly injured podocytes intensely express desmin [40]. We quantified the percentage of glomeruli containing desmin-positive surface podocytes for each mouse. This value was significantly lower in *Keap1^{loxP/-}/Nep25* mice than in *Keap1^{+/+}/Nep25* mice (median 24.5% versus 85.8%) (Figures 5 and 6). Podocyte injury was also assessed by nephrin staining, a more sensitive marker. Nephrin staining was semiquantified

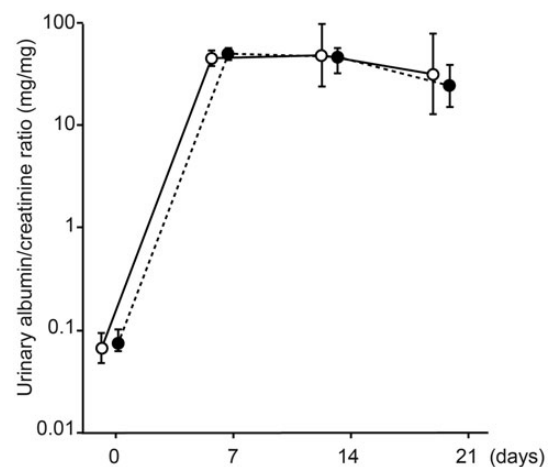


FIGURE 4: Urinary albumin/creatinine ratio. Both *Keap1^{loxP/-}/Nep25* (closed circle) and *Keap1^{+/+}/Nep25* (open circle) mice showed moderate albuminuria, which peaked 1 week after the injection. The degree of albuminuria was not statistically significant between the two mouse groups. Data were presented as geographic means and 95% confidence intervals.

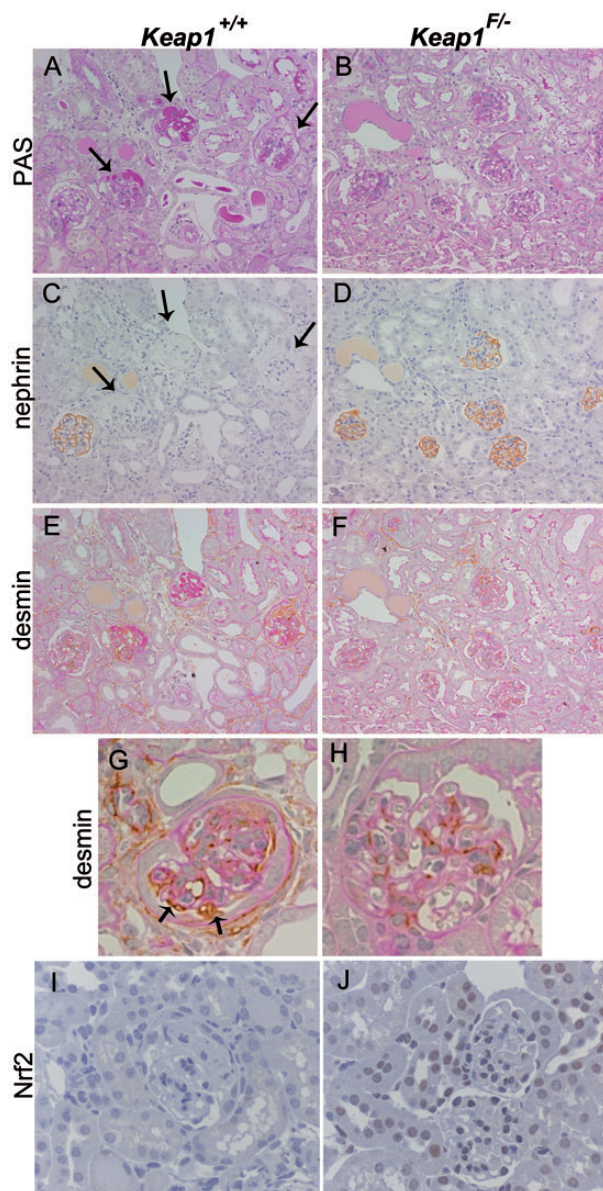


FIGURE 5: Representative pictures of *Keap1*^{+/+}/*Nep25* and *Keap1*^{loxP/-}/*Nep25* (*Keap1*^{F/-}/*Nep25*) after injection of LMB2. *Keap1*^{+/+}/*Nep25* mice showed severe glomerulosclerosis in PAS staining (A, arrows), marked decrease in nephrin staining (C, arrows), and the presence of desmin-positive surface podocytes (E, G, arrows). In contrast, *Keap1*^{F/-}/*Nep25* mice displayed mild glomerulosclerosis (B), almost normal nephrin staining (D), and no desmin-positive surface podocyte (F, H). (I) and (J) show immunostaining for Nrf2. The nuclei in glomerular and tubular cells are positive for Nrf2 staining in *Keap1*^{F/-}/*Nep25* mice (J), but not in *Keap1*^{+/+}/*Nep25* mice. (A), (C) and (E) or (B), (D) and (F) are from adjacent sections. (A–F) ($\times 200$), (G–J) ($\times 400$).

using scores of 0 (no staining) to 8 (normal staining). Nephrin staining was also significantly preserved in *Keap1*^{loxP/-}/*Nep25* mice with a median nephrin index of 6.76, compared with *Keap1*^{+/+}/*Nep25* mice with a median nephrin index of 0.91 (Figures 5 and 6).

Moreover, to evaluate the degree of kidney injury more objectively, we performed real-time RT-PCR assay and

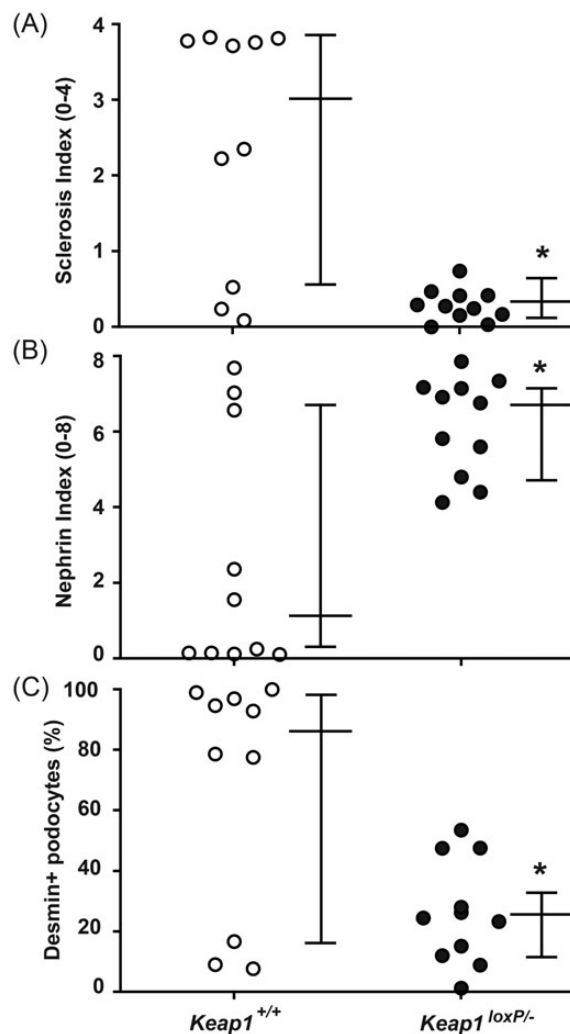


FIGURE 6: Knockdown of *Keap1* gene attenuated the progression of podocyte injury and glomerulosclerosis. Three weeks after LMB2 injection, *Keap1*^{loxP/-}/*Nep25* (*Keap1*^{F/-}/*Nep25*) mice (closed circles) showed significantly attenuated sclerosis index (A), more preserved nephrin staining index (B) and less percentage of glomeruli containing desmin-positive injured podocyte(s) (C), when compared with control *Keap1*^{+/+}/*Nep25* mice (open circles). The horizontal bars represent the medians and interquartile ranges. **P* < 0.05 compared with *Keap1*^{+/+}/*Nep25* mice.

quantified mRNAs of injury-related genes. The results showed that the levels of *Fn1* (the gene for Fibronectin-1; 49.8%), *Tgfb1* (the gene for Tgf- β 1; 62.9%), *Col4a4* (the gene for Collagen, type IV, alpha 4; 61.7%) and *Col1a2* (the gene for Collagen, type I, alpha 2; 56.8%) mRNA expression were significantly lower in *Keap1*^{loxP/-}/*Nep25* mice than in *Keap1*^{+/+}/*Nep25* (Figure 7).

An independent experiment with the same protocol was performed using five *Keap1*^{loxP/-}/*Nep25* mice and eight *Keap1*^{+/+}/*Nep25* mice. Again, *Keap1*^{loxP/-}/*Nep25* mice showed less glomerulosclerosis, less desmin-positive podocytes and more preserved nephrin staining—substantially identical results (Supplementary Figure S4). These results indicate that knockdown of *Keap1* gene attenuated the progression of podocyte injury and glomerulosclerosis.

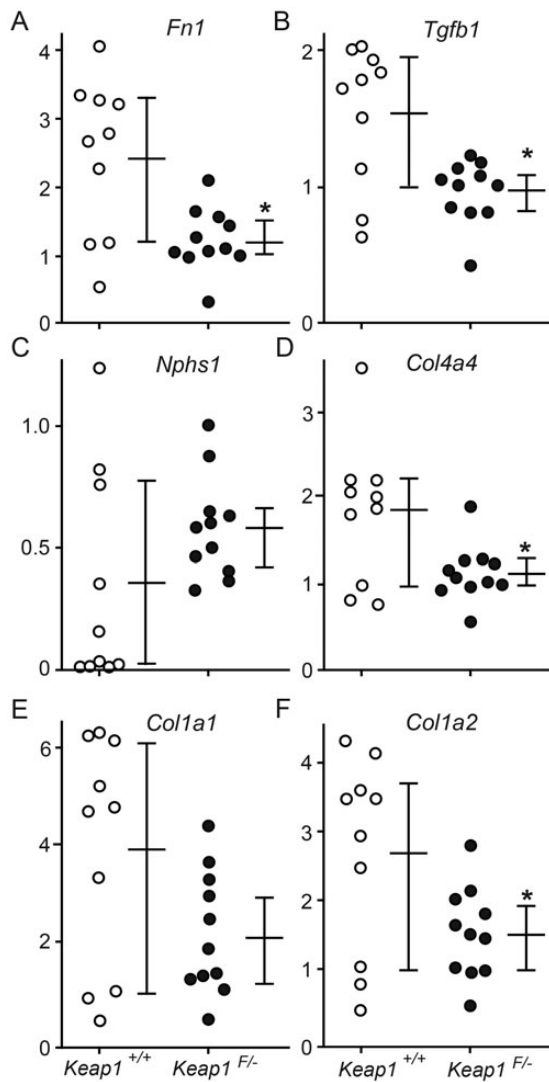


FIGURE 7: Knockdown of *Keap1* attenuated the levels of mRNA for injury-related genes. *Keap1^{loxP/-}* (*Keap1^{F/-}*) kidneys showed significantly less amounts of mRNA for *Fn1* (Fibronectin-1) (A), *Tgfb1* (Tgf- β 1) (B), *Col4a4* (Collagen type IV alpha 4) (D) and *Col1a2* (Collagen type I alpha 2) (F) when compared with *Keap1^{+/+}* kidneys. The levels of *Nphs1* (Nephrin) mRNA (C) and *Col1a1* (Collagen type I alpha 1) mRNA (E) were not significantly different between the two groups, although *Keap1^{F/-}/Nep25* mice show a tendency of protection. *P < 0.05 compared with *Keap1^{+/+}/Nep25* mice.

Immunostaining revealed that nuclear Nrf2 staining was present in some glomeruli and tubules in *Keap1^{loxP/-}/Nep25* mice, but not in *Keap1^{+/+}/Nep25* mice, after LMB2 injection (Figure 5I and J).

Effect of Nrf2 knockout on the progression of glomerulosclerosis

We next examined the effect of *Nrf2* knockout on podocyte injury and glomerulosclerosis. *Nrf2^{-/-}/Nep25* and *Nrf2^{+/-}/Nep25* mice were treated with 0.625 ng/g BW of LMB2 and similarly analyzed. Both types of mice similarly showed moderate albuminuria (Supplementary Figure S5), with no significant difference at any time points. Histological examination at

3 weeks revealed that, although there was a tendency for *Nrf2^{-/-}/Nep25* mice to show more severe glomerular injury, the degree of glomerulosclerosis, nephrin staining index and percentage of desmin-positive podocytes were not significantly different between *Nrf2^{-/-}/Nep25* and *Nrf2^{+/-}/Nep25* mice (Supplementary Figure S6).

DISCUSSION

In the present study, we demonstrated that oxidative stress was induced and systemic knockdown of *Keap1* attenuated glomerulosclerosis and podocyte injury in a NEP25 mouse model. Although the beneficial effect was modest, it was reproducibly observed in two independent experiments. This indicates that oxidative stress is involved in the development of glomerulosclerosis.

It had been shown previously that reactive oxygen species (ROS) are increased in the glomerulus of a puromycin aminonucleoside nephritis (PAN) model, and that proteinuria and morphological changes in podocytes in this model are attenuated by decreasing ROS [41–45]. The NEP25 model, which was used in the present study, is basically a focal segmental glomerulosclerosis (FSGS) model, as opposed to the PAN model, which is a minimal change nephropathy model. LMB2, the recombinant immunotoxin used in the present study, is composed of the Fv portion of the anti-hCD25 antibody combined with the *Pseudomonas aeruginosa* exotoxin PE38. LMB2 itself, unlike adriamycin [32], is not reported to directly generate oxygen radicals. Nonetheless, oxidative stress was modestly increased in the NEP25 kidney, probably by secondary mechanisms. Notably, injected LMB2 is rapidly cleared from the circulation, whereas the podocyte injury progresses over several weeks. Our previous study with chimeric mice indicated that podocyte damage caused by LMB2 secondarily damages other podocytes within the same glomerulus [40]. We therefore speculate that the glomerular phenotype observed 3 weeks after the injection of this low dose (0.625 ng/g BW) of LMB2 reflects both primary and secondary podocyte injury. In that case, the protective effect of *Keap1* knockdown may be universally observed in all glomerular diseases with podocyte depletion.

Nrf2-Keap1 is the master regulatory system of antioxidant genes, and, therefore, an attractive drug target. Since high glucose induces ROS production [46, 47] and ROS initiate podocyte apoptosis [48, 49], it is expected that Nrf2 activators have a beneficial effect on diabetic nephropathy. Indeed, pharmacological activation of Nrf2 has been demonstrated to attenuate metabolic disturbance and albuminuria in streptozotocin-induced diabetic nephropathy [30]. Recently, bardoxolone methyl [50–52], a potent Nrf2 inducer, has been shown to elicit significant improvements in the estimated glomerular filtration rate (eGFR) in type 2 diabetes patients with chronic kidney disease in a phase II clinical trial (BEAM study) [53]. In this study, eGFR was elevated within 4 weeks, accompanied with an increase in urinary albumin-creatinine ratio. Renal histology was not evaluated. This raises the possibility that the improvement of renal function is merely attributed to a

hemodynamic change, but not to the protection of the underlying glomerular architecture. On the other hand, our study showed that the upregulation of Nrf2 target genes indeed attenuates the development of glomerulosclerosis, indicating that Nrf2 activation can have a therapeutic potential commonly for chronic kidney diseases with various etiology [54, 55].

As expected, western blot and real-time PCR analyses confirmed that nuclear Nrf2 and its target antioxidant genes were upregulated in the glomerulus of *Keap1* knockdown mice at baseline. Some *Nrf2* target genes were not upregulated, probably reflecting the diversity of the regulatory mechanism among cell types [35]. Notably, the present study showed that the amount of nuclear Nrf2 in normal glomeruli of wild-type mice was lower than that in the liver. In accordance with this observation, podocyte injury *per se* did not upregulate the antioxidant genes in the glomerulus of wild-type *Keap1* mice for at least 5 days. In addition, podocyte injury did not further upregulate the antioxidant genes in *Keap1* knockdown mice. In this regard, the glomerulus does not appear to be able to quickly respond to oxidative stress evoked by LMB2-induced podocyte injury.

Keap1 knockdown raised the level of nuclear Nrf2 and enhanced the expression of downstream genes in glomeruli. Immunostaining indicated that Nrf2 was also activated in tubule. Since oxidative stress is induced after podocyte injury, the enhanced antioxidative activities may be the major protective mechanism of *Keap1* knockdown, although other mechanisms, e.g. such as the activation of murine double minute (MDM)-2 by Nrf2 [56], cannot be ruled out.

We did not observe that *Nrf2* knockout exaggerated podocyte injury and glomerulosclerosis. This negative result may be due to the small number of mice used in the study. Alternatively, this may be explained by low Nrf2 basal activity and ineffective induction of podocyte injury by LMB2 in glomeruli. Absence of the low level of glomerular Nrf2 may not have a great impact on glomerular injury caused by LMB2. However, previous studies reported that *Nrf2* knockout exaggerated functional or structural injury in mouse DM nephropathy model [29, 57]. The different results may be because the degree of oxidative stress induced by LMB2 is relatively modest compared with the intense stress resulting from hyperglycemia and because the time for disease development in NEP25 mice is relatively short (3 weeks) when compared with that in the DM model (10–16 weeks).

Despite the reproducible attenuation of glomerulosclerosis in *Keap1* knockdown mice, the degree of albuminuria was appreciably indistinguishable between the two types of mice. This is contrasting to the effect of a high dose of losartan, an angiotensin type 1 receptor antagonist, which attenuated both proteinuria and glomerulosclerosis, presumably by reducing glomerular ultrafiltration pressure difference [34]. The Nrf2 activator bardoxolone methyl has been shown to decrease the expression of megalin, a protein involved in the tubular reabsorption of albumin, leading to an increase in albuminuria in monkeys [38]. Therefore, the degree of urinary albumin excretion could be assumed to be overestimated in *Keap1* knockdown mice. However, we found that *Keap1* knockdown

mice similarly express megalin mRNA and protein, and similarly reabsorb albumin to control mice. This indicates that albuminuria similarly reflects glomerular barrier dysfunction in the two mouse groups. The discrepancy in the megalin response between the two studies may be because of the differences between monkeys and mice or because of a possible off-target effect of bardoxolone methyl.

Discrepancy between glomerulosclerosis and proteinuria is often seen in the clinical setting. In this regard, Yu *et al.* [58] reported that proteinuria is not always in parallel with podocyte loss. Indeed, rats with anti-Thy1.1 nephritis showed transient urinary podocyte loss, whereas proteinuria persisted after urinary podocytes disappeared. We speculate that the kidney of *Keap1* knockdown mice is in a similar situation. Thus, *Keap1* knockdown may protect podocytes from severe injury that leads to podocyte loss and glomerulosclerosis, but cannot completely preserve their barrier function.

In conclusion, we have demonstrated that Nrf2 activation by genetic *Keap1* knockdown attenuated glomerulosclerosis in a mouse model of podocyte-specific injury. This study can provide a foundation for the therapeutic use of Nrf2 activators to prevent the progression of renal failure commonly in chronic kidney diseases with various etiologies.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://ndt.oxfordjournals.org>.

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CONFLICT OF INTEREST STATEMENT

None declared.

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Impaired renal growth hormone JAK/STAT5 signaling in chronic kidney disease

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ABSTRACT

Background. Treatment with recombinant human growth hormone (GH) is the standard therapy for short stature in children with chronic kidney disease (CKD). However, concerns have been raised on the potential renal fibrogenic effects of GH. There is no information regarding the renal GH receptor (GHR)-JAK-STAT signaling pathway in CKD.

Methods. Subtotal nephrectomized (CKD) and pair-fed sham-operated control (C) juvenile rats were treated with subcutaneous GH or saline for 2 weeks. A single intravenous GH bolus or vehicle was provided prior to euthanasia.

Results. Reduced body weight in CKD was improved with GH therapy. The remnant kidney showed glomerular hypertrophy and early interstitial fibrosis without inflammatory infiltration. Treatment of CKD rats with GH did not worsen renal function or fibrosis. Kidney GHR mRNA and protein levels were

reduced and basal phosphorylation of JAK2 and STAT5 was significantly impaired. However, intravenous GH administration prior to sacrifice normalized STAT5 phosphorylation. Basal renal IL6 mRNA and phosphorylation of its downstream signaling molecule STAT3 were increased as was the product of its action, the suppressor of cytokine signaling 3 (SOCS3) mRNA.

Conclusions. Despite known unaltered circulating GH levels, remnant kidneys of uremic growth retarded juvenile rats show impaired basal signaling along the GH-activated JAK2/STAT5 signaling pathway. This may well be a consequence of the reduced GHR level and the inhibitory effect of the increase in IL-6-mediated SOCS3 expression. This renal GH insensitivity, if present in humans, may protect against the potential adverse renal effects of GH administration in CKD patients.

Keywords: chronic kidney disease, growth hormone, IL-6, SOCS3, STAT5